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ENDOCRINOLOGY

FELS, I. GORDON, MIRIAM E. SIMPSON, ARNE SVERDRUP AND HERBERT M. EVANS
Enzymatic Digestion of Beef Thyrotropic Hormone with Papain..... 349

SWINGLE, W. W., GEORGE BARLOW, ELLIOTT COLLINS, E. J. FEDOR, WILLIAM J. WELCH AND J. MERCER RAMPONA
Serum Potassium and Electrocardiographic Changes in Adrenalectomized Dogs Maintained on Cortisone Acetate..... 353

NICHOLS, JOHN AND H. L. SHEEHAN
Effect of Partial Adrenal Cortical Atrophy on the Course of Alloxan Diabetes 362

CSAPO, ARPAD I. AND GEORGE W. CORNER
The Antagonistic Effects of Estrogen and Progesterone on the Staircase Phenomenon in Uterine Muscle..... 378

DI STEFANO, H. S., A. D. BASS, H. F. DIERMEIER AND J. TEPPERMAN
Nucleic Acid Patterns in Rat Liver Following Hypophysectomy and Growth Hormone Administration..... 386

SCHRICKER, JOHN ADAMS, GEORGE ROSS FISHER, WILLIAM W. TULLNER AND ROY HERTZ
The Effect of Amphenone-B on the Cholesterol and Ascorbic Acid Content of the Rat Adrenal..... 394

FRIEDMAN, SYDNEY M., CONSTANCE L. FRIEDMAN AND MIYOSHI NAKASHIMA
The Hypertensive Effect of Compound F Acetate (17-OH-Corticosterone-21-Acetate) in the Rat..... 401

LIPNER, H. J., S. B. BARKER AND T. WINNICK
The Distribution of Thyroxine in Rat Liver Cell Fractions..... 406

PORTER, J. C. AND R. M. MELAMPY
Effects of Testosterone Propionate on the Seminal Vesicles of the Rat..... 412

HELMER, O. M. AND R. S. GRIFFITH
The Effect of the Administration of Estrogens on the Renin-substrate (Hypertensinogen) Content of Rat Plasma..... 421

ALBERT, A. AND F. RAYMOND KEATING, JR.
The Role of the Gastrointestinal Tract, Including the Liver, in the Metabolism of Radiothyroxine..... 427

SARTORIUS, OTTO W., DOROTHY CALHOON AND ROBERT F. PITTS
The Capacity of the Adrenalectomized Rat to Secrete Hydrogen and Ammonium Ions..... 444

LEE, NORMAN D. AND ROBERT H. WILLIAMS
The Role of the Pituitary-Adrenal System in Cystine-S^{35} Incorporation into Protein..... 451

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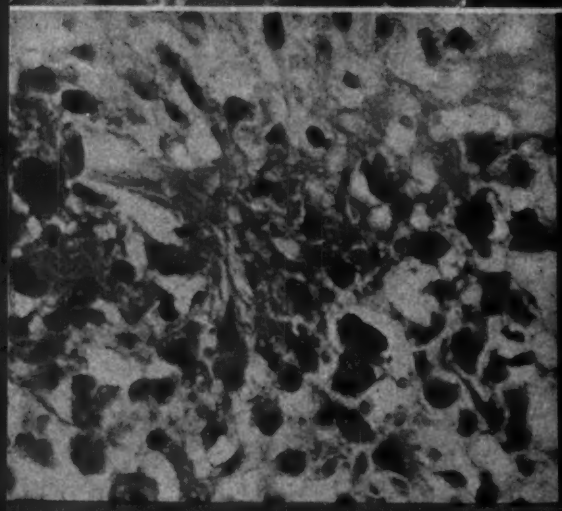
response in rheumatic fever

q. Does cortisone influence the heart lesions of rheumatic fever?

a. Early cortisone administration suppresses and in some cases may even prevent serious cardiac damage.



q. What effect does cortisone have on acute rheumatic fever?

a. Often within 24 hours after cortisone therapy, the severely ill, toxic patient appears alert and comfortable; and within one to four days, temperature drops to normal, appetite increases, and polyarthritis subsides.

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ENZYMATIC DIGESTION OF BEEF THYROTROPIC HORMONE WITH PAPAIN

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IN A STUDY of the effect of a different proteolytic enzymes on a pituitary extract, Chow and co-workers (1939) reported that digestion with papain had no effect upon the thyrotropic activity of the extract. Since no attempt was made to separate the digestion products from the unreacted material, the digestion mixture being injected into the experimental animals, it is difficult to assess the significance of their observation. The present communication reports the action of papain on highly purified beef thyrotropic hormone (TSH). The reaction products have been separated from the undigested material both by trichloracetic acid precipitation and dialysis. In each case it has been found that these products possess thyrotropic activity.

EXPERIMENTAL METHODS

We have found it convenient to use the Long-Evans hypophysectomized, immature female rat as our assay animal. These animals weighed about 70 gms. at the onset of the experiment, and were one week post-operative. Throughout the experiment, they were maintained on an adequate diet (modified McCollum's Diet, No. 1). Solutions to be tested were injected intraperitoneally for three successive days, 0.5 ml. per day, and the animals sacrificed 24 hours after the last injection. The thyroids were excised, fixed, stained, and examined. The lowest concentration of hormone which could affect a discernible histological response in the thyroids of all the animals was considered the minimum effective dose (M.E.D.). This response is characterized by an appreciable change in the height of the epithelium of the acinar cells and initiation of absorption of colloid. At the termination of each experiment animals were not only sacrificed for an examina-

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[1] International Exchange Student.

tion of the thyroid changes but completeness of the hypophysectomy was ascertained. Any animals exhibiting incompleteness were eliminated from the records of the experiments.

The thyrotropic hormone was prepared by a procedure differing somewhat from that reported in the literature and it is hence given in some detail. All operations were conducted at 0–5° C.

Preparation of Thyrotropic Hormone

One kilogram of beef anterior lobe pituitaries was finely ground with frozen CO_2 in an electric grinder. The CO_2 was allowed to sublime away, and the mass was extracted overnight with constant stirring with 10 liters of 0.25% acetic acid—1% NaCl mixture, (Fraenkel-Conrat *et al.*, 1940). The supernatant was siphoned off and the extraction repeated with another 10 liters of the same solvent. The supernatants were collected, and solid $(NH_4)_2SO_4$ added to a 3 molal concentration,[2] with stirring. The precipitate which formed after 24 hours of standing was removed by siphoning and centrifugation. The 3 molal precipitate was dissolved in 4 liters of 1% NaCl, and solid $(NH_4)_2SO_4$ added to make 0.6 molal. The precipitate formed overnight was removed, and to the supernatant sufficient solid $(NH_4)_2SO_4$ was added to bring the concentration back to 3 molal. The precipitate formed after final settling was collected and again dissolved in 4 liters of 1% NaCl. This solution was made up to 1.2 molal with solid $(NH_4)_2SO_4$. Complete precipitation occurred after 1½ hours, whereupon the supernatant was removed and brought up to 2 molal with $(NH_4)_2SO_4$. The 2 molal precipitate after collection was dissolved in 250 ml. H_2O and dialized in the cold for two days with frequent changes of water. To the salt free solution cold acetone (−10° C.) was added slowly with stirring to a final concentration of 39%.

The mixture was allowed to stand for ½ hour after which time it was centrifuged and the supernatant poured into ten times its volume of cold acetone. After 2–4 hours the precipitate was collected at the centrifuge, washed three times with cold acetone and finally with cold anhydrous ether. The material which was dried in a vacuum desiccator is a white powder weighing about 1.5 gms. At the 1 mg. level it is free of growth hormone using the rat tibia test (Greenspan *et al.*, 1949), of ACTH using the adrenal repair test (Simpson *et al.*, 1943), of FSH and ICSH using the follicle growth test (Evans *et al.*, 1939), and the interstitial cell repair method (Simpson *et al.*, 1942) respectively. It is also free of lactogenic hormone at the same level, using the minimum stimulation method in pigeons (McShan *et al.*, 1936). The thyrotropic hormone obtained by this method has a minimum effective dose of 25 micrograms according to the assay described above.

Digestion with Papain

About 0.5 gm. of purified thyrotropic hormone was dissolved in 50 ml. of 0.25 M acetate buffer, pH 4.7. To this was added 200 mg. commercial papain[3] activated with 30 mg. cysteine hydrochloride in 50 ml. .05 M acetate buffer, pH 4.7. Immediately upon mixing, two 5 ml. aliquots were removed. One was dialyzed (Visking 24/32) at 0° C. for three days against a large volume of cold water, with frequent changes, and was used both for micro-Kjeldahl and biological activity determinations. The low temperature and the loss of the activating cysteine through dialysis were sufficient to depress further enzymatic activity to a minimum. To the other aliquot, an equal volume of cold 40% tri-

[2] Concentration is expressed as molality on the assumption that one liter of solvent prior to the addition of salt at the operational temperature weighs one kilogram.

[3] General Biochemicals, Inc. Laboratory Park, Chagrin Falls, Ohio.

chloracetic acid (TCA) was added slowly, placed in the cold for ½ hour and centrifuged. The supernatant, after three extractions with 50 ml. ether, was used to determine nitrogen and biological activity. The digestion mixture was incubated at 37° C. Aliquots were removed at different intervals, and subjected to the three days' dialysis at 0° C. The percentage hydrolysis was estimated from the nitrogen values of the non-dialyzable residue after dialysis. The results are given in Table 1.

TABLE 1. THE TSH ACTIVITY OF PAPAIN DIGESTED THYROTROPIC HORMONE

Material	Hours digested	mg. N/ml.*	% Hydrolysis	TSH activity†
Non Dialyzable Residue	0	1.32	—	+
	5	1.06	20	+
	12	1.02	23	+
	24	0.97	27	+
Supernatant After TCA Precipitation	0	0.05	—	Negative
	5	0.23	20	+
	12	0.28	23	+
	24	0.28	27	+
Dialysate TSH alone	—	—	—	Negative
Dialysate Digestion Mixture	0	0.48	—	Negative
	5	0.34	29	±
	12	0.32	32	±
	24	0.29	39	+
	48	0.30	37	+

* Average of two Kjeldahl determinations.
† Three hypophysectomized rats per group. A designation of + signifies that all three gave a positive histological response. A designation of ± signifies that one out of three gave a positive response.

In another experiment, 5 ml. aliquots were removed from the digestion mixture as in the procedure above. These were placed in small dialysis bags (Visking) which were double knotted at both ends. The exterior of the bags was thoroughly washed with distilled water, and the bags dialyzed in the cold against 10 ml. distilled water for three days in a small screw top vial. At the end of the dialysis, the dialysates were used directly in testing for TSH activity. A control containing TSH without papain was also dialyzed. The degree of hydrolysis of the digested material was determined as above with separate aliquots which were dialyzed against a large volume of cold water. The results are tabulated in Table 1.

DISCUSSION

The evidence presented from two methods of approach, namely, trichloracetic acid precipitation and dialysis, indicates clearly that the thyrotropic hormone may be enzymatically broken down by papain and still retain its activity.

It is pertinent at this point to ask whether the hormonal activity of TSH resides in a small molecule which in its native state, or as a result of our chemical procedure, is adsorbed on a large protein, or whether the TSH molecule is originally a non-dialyzable protein, having a covalent-bound

prosthetic group, to use the terminology of enzymology. Either case could account for the results obtained here.

Preliminary experiments to elucidate this point have not been conclusive. Thus, material having thyrotropic activity obtained directly from anterior pituitaries by freezing and thawing does not dialyze out of Visking tubing. In addition, if this same material is extracted with a final concentration of 5% trichloracetic acid, the acid of the extract removed with ether, and the solution dialyzed, all the activity is retained in the dialysis bag. This is the procedure used by Geschwind *et al.* (1950) in isolating dialyzable adrenalcorticotrophic active material. These workers similarly were unable to demonstrate any dialyzable TSH activity using this method

On the other hand, using electrodialysis with goldbeater's skin as a positive membrane, some active material can be made to pass through a Visking casing membrane in three hours, whereas in ordinary dialysis no penetration has been observed after 5 days. Attempts to increase the porosity of the membrane with surface active materials such as Bactopeptone and sodium tauroglycolate (Brinkman and Szent-Gyorgyi 1923) have not affected the passage of the hormone.

SUMMARY

(1) The digestion of purified thyrotropic hormone with papain results in biologically active, dialyzable material which is not precipitated by a final concentration of 20% trichloracetic acid. The implication of these results is discussed.

(2) A purification procedure for thyrotropic hormone is given. The hormone is free of ACTH, FSH, ICSH, growth hormone and lactogenic hormone at the 1 mg. level in the hypophysectomized rat.

Acknowledgement

The technical assistance of Mrs. Clara Stern is gratefully acknowledged.

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SERUM POTASSIUM AND ELECTROCARDIOGRAPHIC CHANGES IN ADRENALECTOMIZED DOGS MAINTAINED ON CORTISONE ACETATE[1]

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DURING the course of certain long-term experiments on adrenalectomized dogs maintained on varying doses of cortisone (Swingle, Collins, Barlow and Fedor, in press), it was repeatedly observed that some of the animals, although appearing active, vigorous and free from symptoms of any kind, would suddenly collapse and remain prostrate for periods ranging from five minutes to several hours. The animals retained consciousness and seemed free from pain but the limbs, especially the hind ones, appeared to be paralyzed. Recovery from the attacks was spontaneous and usually required no special treatment. Occasionally an animal would experience repeated attacks of prostration within a few hours. Eventually the untreated animal died during one of the episodes. Intravenous infusion of one per cent saline, or 300 mg. of the water-soluble glucoside of desoxycorticosterone, or cortical extract caused prompt disappearance of symptoms. Also, doubling the daily cortisone dosage was effective in most cases in bringing about cessation of the attacks.

Numerous observations of the mineral constituents of the blood revealed that the periods of prostration coincided with high levels of serum potassium. It was concluded that the cause of the sudden collapse of these animals was probably hyperpotassemia affecting cardiac function.

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[2] Research Fellow of the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

A part of this paper is based on a dissertation presented by George Barlow to the Graduate School of Princeton University in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

It should be understood, however, that none of the adrenalectomized dogs reported upon here were displaying signs of insufficiency on the day of collapse; they were strong, vigorous and with one exception, eating full rations. During the interval when they were receiving the lowest dosage of cortisone given in these experiments, i.e., 0.93 mg./kg./day, the animals maintained mean arterial pressures within the normal or low normal range. However, they exhibited a drastic decline in serum Na and Cl and a sharply elevated serum K. Adrenalectomized dogs receiving inadequate maintenance doses of cortical extract or DCA, and exhibiting similar alterations of serum electrolytes as did these cortisone-maintained animals, would undoubtedly have shown typical gross symptoms of adrenal insufficiency. Thus, we emphasize the fact that despite the low serum Na and Cl and high serum K levels, the cortisone-treated dogs displayed surprising strength, vigor and appetite. They were indistinguishable from unoperated animals.

In an earlier paper, Swingle, *et al.* (in press) called attention to the fact that if it were not for the slow accumulation of serum K to a concentration which interfered with proper functioning of the heart, adrenalectomized dogs could probably be maintained indefinitely at low serum Na and Cl levels. We have kept such animals for as long as 89 days on a daily cortisone dosage of 0.93 mg./kg./day before collapse from hyperpotassemia occurred.

Several groups of investigators have made electrocardiographic studies of adrenalectomized dogs exhibiting high serum K when showing symptoms of insufficiency (Nicholson and Soffer, 1935; Hall and Cleghorn, 1938; and Roberts, 1952). Similar studies have also been made on Addisonian patients during crisis and recovery or while maintained on various types of therapy, e.g., salt, DCA in oil, cortical extract and implanted DCA pellets (Sampayo, *et al.*, 1934; Mattioli, *et al.*, 1937; Thorn, *et al.*, 1942; Soffer, 1946).

Recently, Roberts (1952) has reported on electrocardiographic changes occurring in the adrenalectomized dog in insufficiency. Injections of cortisone plus NaCl or NaCl alone corrected the abnormalities appearing on the tracings of the untreated animals. Cortisone alone was ineffective if given when gross symptoms were present. No dosage figures were given for cortisone.

In the experiments recorded here, long-term studies were made of cortisone-treated adrenalectomized dogs followed during the slowly developing hyperpotassemia which invariably occurs when adrenalectomized dogs are maintained for long periods solely on 0.93 mg./kg./day. Several animals were also studied through 120 days of cortisone therapy during

which time sharp, stepwise increases in dosages were found necessary to prevent dangerous accumulation of serum K. But three dogs in our colony of 16 animals have required cortisone dosage in excess of 1.86 mg./kg./day for adequate maintenance, not only for activity and vigor but also for maintenance of normal levels of the blood constituents.

METHODS

Seven adult, healthy mongrel dogs, six males and one female, were adrenalectomized and kept for varying intervals before use in these experiments. Some animals had been operated for 12–18 months, others for lesser periods. When ready for use, they were fed a special diet consisting of horse meat, tripe and lard, plus small amounts of brewer's yeast and the necessary vitamins. The total dry weight of the daily ration fed each animal was 290 gms. plus 145 cc. of tap water added to make a thick mush. This ration contained a total of 0.93 gm. of sodium and 0.94 gm. of potassium. In previous publications the daily ration was reported as 430 grams but this amount has since been reduced one-third owing to rapid weight gain of the dogs. Cowgill's (1923) modified salt mixture containing neither Na nor K was added to the food each day.

The methods used for blood pressure determinations and the various blood analyses have been referred to elsewhere (Swingle, Perlmutt, Seay and Collins, in press) and need not be repeated here.

Merck's Cortone, in fine crystalline suspension, was used and was administered intramuscularly once daily. The starting dose for all of the dogs was 0.93 mg./kg./day. This was a purely arbitrary figure and proved inadequate for maintaining a normal serum electrolyte pattern but, nevertheless, was sufficient to keep the dogs strong and active. When the serum K accumulated to such an amount that there was a strong probability of the animal collapsing from cardiac failure, the daily dose of cortisone was stepped up to 1.86 mg./kg./day. This dose was again doubled to 3.72 mg./kg./day whenever the serum K climbed to more than 7.5 m.eq./l. This quadrupling of the starting dose proved to be necessary for only three dogs. No attempt was made to maintain the animals on cortisone for more than 120 days. Standard leads I, II and III were recorded serially with a direct writing instrument. The illustrations show lead II only and have been retouched. All dogs were table trained for blood pressure determinations and electrocardiographic studies.

EXPERIMENTAL RESULTS

The data obtained from the 7 dogs were quite similar and the tracings are practically identical in all cases; hence, but one animal, typical of the group, will be discussed in detail (Table 1). The essential data obtained from study of all of the dogs are presented in Table 2.

Animal No. 1, representative of the series, was maintained on cortisone for 120 days after which he was placed on a regime of DCA in oil. This animal is in excellent health at the present time. Electrocardiograms were taken at frequent intervals throughout the experimental period. The pertinent facts regarding this dog are given in Table 1, and in the ECG tracings, *A* through *G*, of Figure 1.

The dog, which had been adrenalectomized several weeks previous and maintained on small doses of DCA,[3] received an initial injection of cortisone representing 0.93 mg./kg./day on 12/4/51. Tracing *A* is the control ECG record taken on this date. The serum K was 4.69 and the serum Na 143 m.eq./l. The mean arterial pressure registered 128 mm. Hg. The tracing shows a normal sinus rhythm. The negativity of the T wave is the

TABLE 1. ELECTROCARDIOGRAPHIC CHANGES IN AN ADRENALECTOMIZED DOG MAINTAINED ON CORTISONE FOR 120 DAYS AND EXHIBITING HYPERPOTASSEMIA AND RECOVERY

Cortisone dosage mg./kg./day	Duration days	Blood pressure mm. Hg.	Blood urea N m. %	Serum Na m.eq./l	Serum K m.eq./l	ECG records
Control period	0	128	19.4	143.1	4.69	Normal inverted T waves due to position of heart.
0.93	48	95	38.0	134.6	7.60	T waves of shorter duration and increased amplitude. ST segments depressed.
	53	112	29.3	129.0	8.35	Above changes more marked.
	59	90	38.9	140.0	9.80	Further depression of ST segment.
1.86	10	112	17.5	146.0	5.39	ST segments back to base line. T waves still deeply inverted.
	20	108	24.5	135.7	7.11	Further increase in amplitude of T waves (6 mm.).
	28	119	36.0	144.6	7.87	No change.
3.72	8	107	12.6	147.9	4.69	Normal tracing.
	32	118	12.7	147.4	5.12	No essential change.

result of the position of the heart in the chest. According to Hall and Cleghorn (1938), the normal T wave in the dog may be positive, negative or diphasic.

Forty-eight days following the initial injection and while the dog was receiving the same dosage, the blood pressure had declined to 95 mm. Hg, the serum Na had fallen to 134.6 and the serum K had risen to 7.6 m.eq./l. Despite the severe alterations in electrolyte pattern, the dog was active, vigorous and exhibited no indications of adrenal insufficiency. Tracing *B* is an ECG taken at this time and shows a depression of the ST segment and a decrease in the duration of the T wave. Five days later, i.e., 53 days from

[3] We are indebted to the Ciba Pharmaceutical Products Co., Inc., for the Percorten and Percorten Glucoside and to Merck & Co., Rahway, N. J., for generous supplies of Cortisone Acetate.

TABLE 2. ELECTROLYTE AND ELECTROCARDIOGRAPHIC CHANGES IN ADRENALECTOMIZED DOGS MAINTAINED ON CORTISONE.

Dog no.	Cortisone dosage mg./kg./day	Duration days	Blood pressure, mm. Hg.	Blood urea N, mg. %	Serum Na, m.eq./l	Serum K m.eq./l	ECG records
1A	Control period	0	97	12.3	141.0	4.40	Normal record.
	0.93	45	105	87.0	138.7	7.87	Sinus arrythmia. T waves of short duration and increased amplitude. Dog in collapse.
1B	Control period	0	80	11.8	141.0	4.40	T waves on a broader base, amplitude decreased.
	0.93	14	89	44.4	127.9	8.40	Dog in collapse. Auricular fibrillation. T waves within normal range in lead II and isoelectric in lead I.
	3.72	37	104	12.0	147.8	4.40	Essentially normal.
2	Control period	0	93	16.6	146.2	4.96	Normal record.
	0.93	14	84	24.0	130.0	8.70	High peaked T waves, depression of ST segment.
		35	74	18.0	128.0	8.19	Absence of P waves. High peaked T waves of increased amplitude; depression of ST segments.
	1.86	30	102	18.8	130.7	5.50	T waves decreased from 11 mm. to 6 mm. in height.
3	Control period	0	98	23.0	146.0	4.90	Normal record.
	0.93	10	94	54.0	129.4	7.87	High peaked P waves, depression of ST segments and diphasic T waves.
	1.86	10	105	17.7	138.4	4.85	Normal.
4A	Control period	0	93	10.2	142.8	4.85	Normal record.
	0.93	85	112	33.0	114.5	9.06	High peaked T waves, auricullar fibrillation. Dog collapsed.
	1.86	5	109	32.0	120.0	7.40	Sinus tachycardia. Normal configuration of T waves.
4B	Control period	0	108	12.7	147.0	5.50	Normal record.
	0.93	7	90	39.0	130.0	8.35	Dog in collapse. Auricular fibrillation, depressed ST segments and high spiking T waves.
	1.86	3	—	—	119.0	9.50	Dog in collapse. Died before record was taken.
5	Control period	0	120	12.0	149.0	4.69	Normal record.
	1.86	10	117	21.4	140.0	6.14	High peaked P waves, depression of ST segment, diphasic T waves.
		30	110	24.0	145.8	7.87	T waves sharply inverted.
		60	122	33.2	143.3	7.87	Essentially as above.
	3.72	30	118	10.1	147.0	5.12	Normal tracing.
6	Control period	0	118	15.1	147.0	4.85	Normal record.
	1.86	11	125	21.9	138.0	7.10	Sharp increase in amplitude of T waves with sagging ST segments, P waves are peaked and tall.
		60	132	17.5	138.2	4.40	Partial return toward normal configuration.

the date of initiating the cortisone treatment of 0.93 mg./kg./day, the serum K was 8.35 and the serum Na 129 m.eq./l. The arterial pressure

was 112 mm. Hg but the dog was free of gross signs or symptoms of adrenal insufficiency. Tracing *C* was taken at this time; it shows exaggeration of the abnormalities seen in tracing *B*.

Although the animal was lively and displayed much activity, nevertheless his appetite was failing and three days later (56 days from the start of the experiment), he refused all food and was given 100 cc. of one per

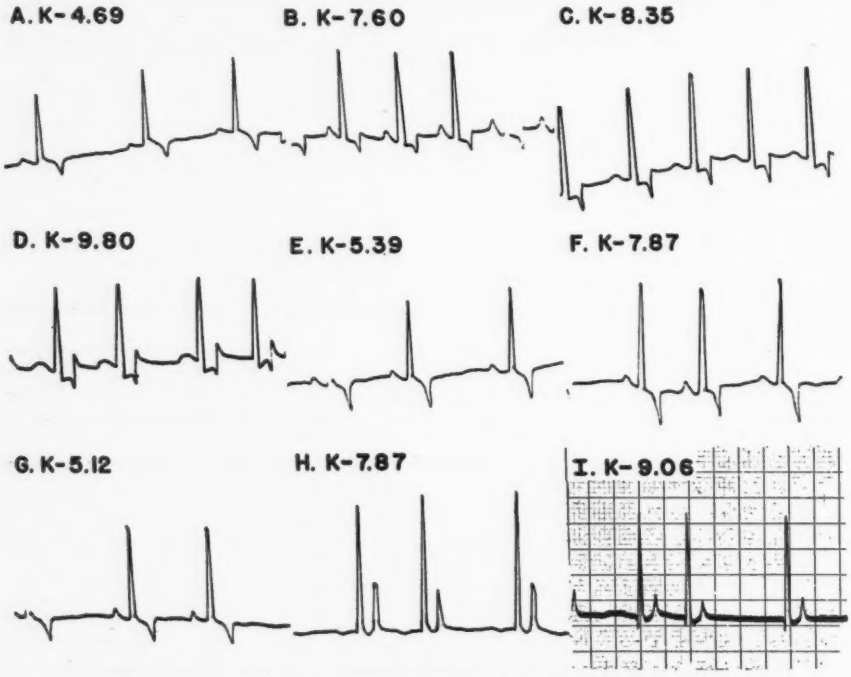

FIG. 1. ECG tracings taken on dogs maintained on cortisone and exhibiting hyperpotassemia and recovery. Potassium figures are in m.eq./l. *A–G* dog 1, Table 1, *H* and *I* are dogs 1A and 4A, respectively, Table 2.

cent saline solution by stomach tube and this was repeated the following day. The dog's appetite returned after the salt administration and he was again placed on the special low sodium diet. He remained on this diet without further salt supplements until the end of the experiment which was approximately 64 days later. This is the only dog of the series that received salt other than that in the diet. On the 59th day, or three days after giving saline, the serum Na was 140 and the serum K was 9.8 m.eq./l. It was anticipated that the serum K would decline with increase in serum Na but such was not the case. This observation has been repeated in other long-term cortisone-treated dogs (Nos. 1A and 5. Dog 6 also shows an

elevated K and normal Na, Table 2). We have encountered but one other dog showing serum K figures with values as high as those attained in this animal, e.g., dog 4A, Table 2.

The ECG record shown in tracing *D* was taken at the height of the hyperpotassemia and reveals several interesting features. Note the broad, flat P wave and further exaggeration of the ST segment depression and short duration of the T wave.

The evidence seemed clear that a daily cortisone dosage of 0.93 mg./kg./day, given as a crystalline suspension, was insufficient to maintain a normal serum electrolyte pattern and, notwithstanding the activity and vigor of the animal, would eventually prove fatal, owing to accumulation of K to such an extent that cardiac failure would result. For example, dog 4B, Table 2, with high serum K, collapsed and died within 10 minutes when merely lifted from his cage. The serum K registered 9.5 m.eq./l.; a few minutes previous to collapse, this dog was jumping about his cage, barking for food, and appeared completely symptom-free.

The daily cortisone dosage on the representative animal under discussion (dog 1, Table 1) was raised from 0.93 to 1.86 mg./kg./day. In general, the higher dose was sufficient to maintain normal serum electrolyte levels. This represents 22.8 mg. of cortisone/day. Aside from dog 1, Table 1, we have observed two other animals, e.g., dog 5, and 1B, Table 2, whose daily cortisone requirement was larger than 1.86 mg./kg./day in order to keep the serum K from increasing to dangerous levels.

Ten days following introduction of the higher dosage regimen, the serum electrolytes, blood pressure and blood urea nitrogen were all normal. An electrocardiogram record taken at this time is shown in tracing *E*. The sharp depression of the ST segment has disappeared and only the increased amplitude of the T wave persists.

Twenty days after administering the increased dosage, the serum K had risen from 5.39 to 7.11 and the serum Na had declined to 135.7 m.eq./l. (Table 1). By the 28th day of higher cortisone dosage, the serum K was 7.87 m.eq./l., the serum Na was normal and the arterial pressure registered 119 mm. Hg. The ECG tracing *F* reflects the increasing K concentration. The amplitude of the T wave had increased from 5 mm. to 8 mm. with an associated decrease in duration.

The dog appeared to be in excellent health and when let out of his cage, would dash around the animal quarters ready to play or fight as the occasion presented. However, since the serum K was at a dangerously high level and slowly increasing, the daily cortisone dose was again raised, this time to four times the original dose, or to 3.72 mg./kg./day. The dog was kept on this dosage for 32 days. The serum Na remained at a normal level and the serum K fell from 7.87 to 4.69 m.eq./l., within 8

days. The ECG tracing at this time is identical with the record shown in tracing *A* taken at the beginning of cortisone therapy.

Dog 1B, Table 2, shows a similar drastic decline in serum K after quadrupling the original cortisone dosage. The same thing may also occur when lower doses are given, e.g., 1.86 mg./kg./day, as shown in Table 2 (dogs 2 and 3). On the other hand, as previously stated, we have encountered dogs which show a slow but steady increase in the serum K even on 1.86 mg./kg./day.

One hundred and twenty days from the beginning of the study, the animal was normal as to activity, vigor, serum electrolytes, blood pressure and urea nitrogen. The final ECG tracing (*G*) of the series was taken at this time. It reveals no abnormalities and is essentially identical with the control record.

The ECG records of two of the cortisone-treated dogs were taken just after they had collapsed and while they were prostrate. These are shown in tracings *H* and *I*. The serum K of both animals was in excess of 7.8 m.eq./l. (dogs 1A and 4A, Table 2). The electrocardiograms reflect the high serum K and show the changes characteristic of hyperkalemia.

DISCUSSION

The ECG records of the cortisone maintained dogs are closely similar, if not identical, with the serial electrocardiograms reported by Winkler, Hoff and Smith (1938), taken during the intravenous infusion of KCl in dogs. The records presented here likewise appear not to differ from those of Nicholson and Soffer (1935), Hall and Cleghorn (1938) and Roberts (1952), for adrenalectomized dogs during episodes of severe adrenal insufficiency.

Although the ECG records taken during adrenal crisis and those recorded here for long-term cortisone-treated dogs exhibiting high serum K levels are similar, the clinical condition of the animals at the time the tracings were taken differed greatly. The cortisone-treated animals did not show symptoms at any time. Even the dogs in collapse due to hyperkalemia usually recovered after brief intervals and were strong and active between the bouts of cardiac distress. The lack of typical symptoms of adrenal failure was surprising in view of the greatly distorted serum electrolyte pattern. As was mentioned earlier, it is extremely unlikely that an adrenalectomized dog not receiving large doses of cortisone could withstand the stress incident to such low serum Na and Cl and high serum K and still retain activity and vigor seemingly unimpaired.

Long-term cortisone therapy in adrenalectomized dogs, except in relatively enormous doses, such as 1.86 mg./kg./day, appears not to prevent the slow accumulation of serum K. Even at this dosage, the impaired

ability of the kidney to eliminate K is not completely restored to normal in some animals. Usually, however, this amount of cortisone given as a crystalline suspension intramuscularly, is adequate to sustain normal levels of all blood constituents. Hence, there can be no doubt that cortisone in sufficient dosage can serve as a complete replacement therapy in adrenalectomized dogs (Roberts and Pitts, 1952). The daily cortisone requirement for maintenance of health, vigor and a normal serum electrolyte pattern, when the steroid is given as a fine, crystalline suspension once daily, is at least 60 times greater than that for DCA in oil.

SUMMARY

Adrenalectomized dogs can be maintained active, vigorous and free from insufficiency symptoms for long periods on a daily cortisone dosage of 0.93 mg./kg./day. This dosage is inadequate for keeping the serum electrolyte pattern normal. The serum Na and Cl decline to levels characteristic of adrenal insufficiency and the serum K becomes markedly elevated. The slow accumulation of serum K eventually becomes so great the animals collapse and may die from cardiac failure unless promptly treated. Study of the ECG of these animals reveals electrocardiographic abnormalities characteristic of hyperpotassemia. With few exceptions, increasing the daily cortisone dosage to 1.86 mg./kg./day corrects the serum electrolyte changes and cardiac deficiencies, and the ECG becomes normal. Animals so treated have been maintained for 120 days solely on cortisone therapy.

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EFFECT OF PARTIAL ADRENAL CORTICAL ATROPHY ON THE COURSE OF ALLOXAN DIABETES*

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IT IS well known that ablation of the adrenal gland (Long-Lukens animal) or of the pituitary (Houssay animal) will ameliorate pancreatic diabetes. The problem arises whether atrophy of part of the adrenal cortex produced by chemical agents might also produce this effect.

The oral administration of DDD (2,2-bis (parachlorophenyl)-1,1-dichloroethane) was found by Nelson and Woodard (1949) to cause atrophy and disappearance of the inner zones of the adrenal cortex in the dog, and it has since been shown (Nichols and Gardner, 1951) that such animals show a degree of insulin sensitivity. These findings have been confirmed in the case of the rat by Brown (1952). If such atrophy of the adrenal should prevent or modify pancreatic diabetes without producing any sensitivity to potassium, the site of production of the hormone(s) responsible for maintenance of the diabetes could be presumptively localized to the inner zones of the cortex, and those responsible for electrolyte balance could be localized to the outer zone. The present paper is the report of an experiment designed to test this hypothesis.

MATERIALS AND METHODS

Eleven mongrel dogs, weighing 5 to 10 kg. each, and four greyhounds, weighing about 20 kg. each, were given DDD in capsules by mouth in daily doses of 50 mg. per kg. Seven of the dogs were treated in this way for 5 weeks and were then used in the alloxan experiments described below. The other eight dogs were killed or died at various times from 3 to 23 days after beginning the DDD treatment. Most of the animals showed no clinical effects from the treatment. Three dogs, at some time during the first 3 weeks, lost their appetite, developed serious muscular weakness and incoordination, and died in coma within 1 or 2 days. Five DDD-treated animals were killed with nembutal at various stages during the first 3 weeks; the dose required to produce death was found to be much less than in a normal dog.

The seven dogs which had been allowed to complete the full course of DDD treatment were then given an intravenous injection of 75 mg. of alloxan (B.D.H.) per kg. in the morning before their daily feed. No further DDD was given to these animals afterwards. Three dogs which had not been treated with DDD were given the same dosage of alloxan, and were studied as controls.

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Blood sugar determinations by Benedict's method (1931) were made immediately before the injection of the alloxan and at frequent intervals afterwards, as shown in Table 1. Two of the DDD-treated dogs died in the hypoglycemic phase after the alloxan.

At 2½ to 5 days after giving the alloxan, the five surviving dogs were subjected to a potassium tolerance test, consisting of the oral administration of 0.5 to 1 gm. of potassium chloride per kg., followed by a similar dose 6 hours later. They were left under observation for one or two days after this test, and were then killed by the intravenous injection of chloroform and autopsied immediately.

Routine histological examinations of tissues were made. In addition, the adrenals were studied in Sudan-stained frozen sections and the islet granules of the pancreas were examined in Zenker-fixed material stained by the method of Gomori (1939).

TABLE 1. BLOOD SUGAR LEVELS IN MG. PER 100 ML. AFTER ALLOXAN. ASTERISKS INDICATE THE TIME AT WHICH INTRAVENOUS GLUCOSE WAS BEGUN

Time after alloxan	Dogs previously treated with DDD							Normal dogs		
	F	M	O	P	Q	R	N	A	B	C
hours										
0	80	87	81	75	80	71	62	60	66	57
½	50	73	91	80	58	95	62	60	50	50
1	50	63	88	100	70	102	80	50	60	53
2	80	63	100	102	80	101	80	160	175	120
4	80	110	93	105	80	67	140	210	233	200
6	88	50	50*	75	42	52	140	250	253	190
8	125	50*		65	45	42	115	260	250	180
10	120			55	45	37		160	200	170
12	62*	55		45*	40*	30	110	200	253	150
14		60			32	27*	100	350	253	200
16		60		47			*	380	200	210
20		83		47	42		25	350	250	87
24								220	227	50*
26		37					35			
28								230	180	33
30								43	52*	12
32								20*	15	
days										
2			100	93	75	100	113	140	113	
3			100	100	145	150	111	300	320	
4			93	125			120	190	250	
5			110				102	240	250	
6			105				117	400	330	
7							110	370	350	
8							100	195	266	

BIOCHEMICAL FINDINGS

The course of the blood sugar following alloxan is shown in Table 1, and in Figs. 1 and 2.

(a) There was an initial brief fall of blood sugar. This was similar in both groups of animals.

(b) Subsequently the control dogs developed a considerable hyperglycemia lasting about a day. The length of this phase in the controls was rather greater than is usually found, presumably depending on

the diet of the animals. The DDD-treated dogs had only a slight rise of blood sugar, and this rise had usually passed off within about 4 to 6 hours.

(c) Both groups of animals then passed into a phase of severe hypoglycemia lasting about 18 hours. In the DDD-treated dogs this phase began earlier and was more profound than in the control dogs. Larger doses of glucose were required to keep these animals alive than the control dogs, and, despite frequent administration of

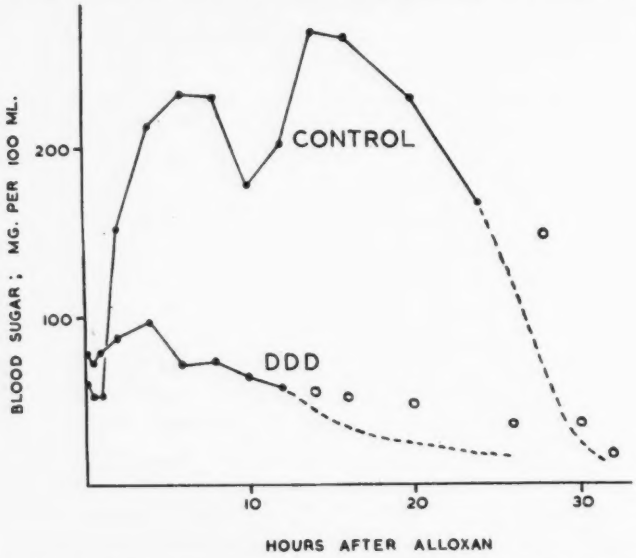

FIG. 1. Mean blood sugar curves during first 30 hours after alloxan, in dogs previously treated with DDD and in control dogs. At the end of each curve, marked by a broken line and by open circles, the dogs were being given large doses of glucose.

large amounts of glucose, two of the DDD-treated dogs died at this stage. One of the control dogs died in this phase, but this death was in the nature of an accident as glucose was not given at the significant time.

(d) Within 2 days the control dogs had the permanent severe hyperglycemia characteristic of alloxan diabetes. In striking contrast, the DDD-treated dogs had fasting blood sugars little if at all raised above normal.

The potassium tolerance test produced none of the untoward effects which occur in adrenalectomized dogs. The animals had a marked polyuria and drank large amounts of water but were otherwise quite well.

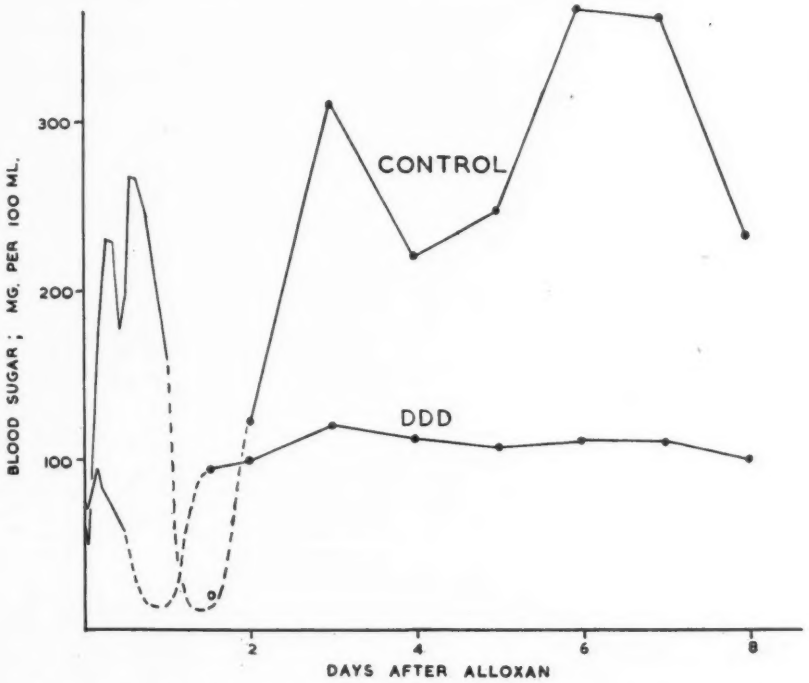

FIG. 2. Mean blood sugar curves during 8 days after alloxan, in dogs previously treated with DDD and in control dogs. The period of hypoglycemia during which the dogs were treated with glucose is indicated by a broken line.

HISTOLOGICAL FINDINGS

Adrenals

There were two kinds of histological change in the adrenal cortex: atrophy and cellular infiltration.

Atrophic changes

At 3 to 5 days after beginning the DDD treatment, the cells of the zona fasciculata and zona reticulata had excessive vacuolation of their cytoplasm due to large droplets of lipoid which stained very brightly with Sudan III. The nuclei of these cells were normal. The zona reticulata showed considerable congestion. In a few non-vacuolated cells in the outer half of the zona fasciculata, the nuclei had the histological appearances of the metaphase of mitosis but no actual division was observed. The zona glomerulosa was normal.

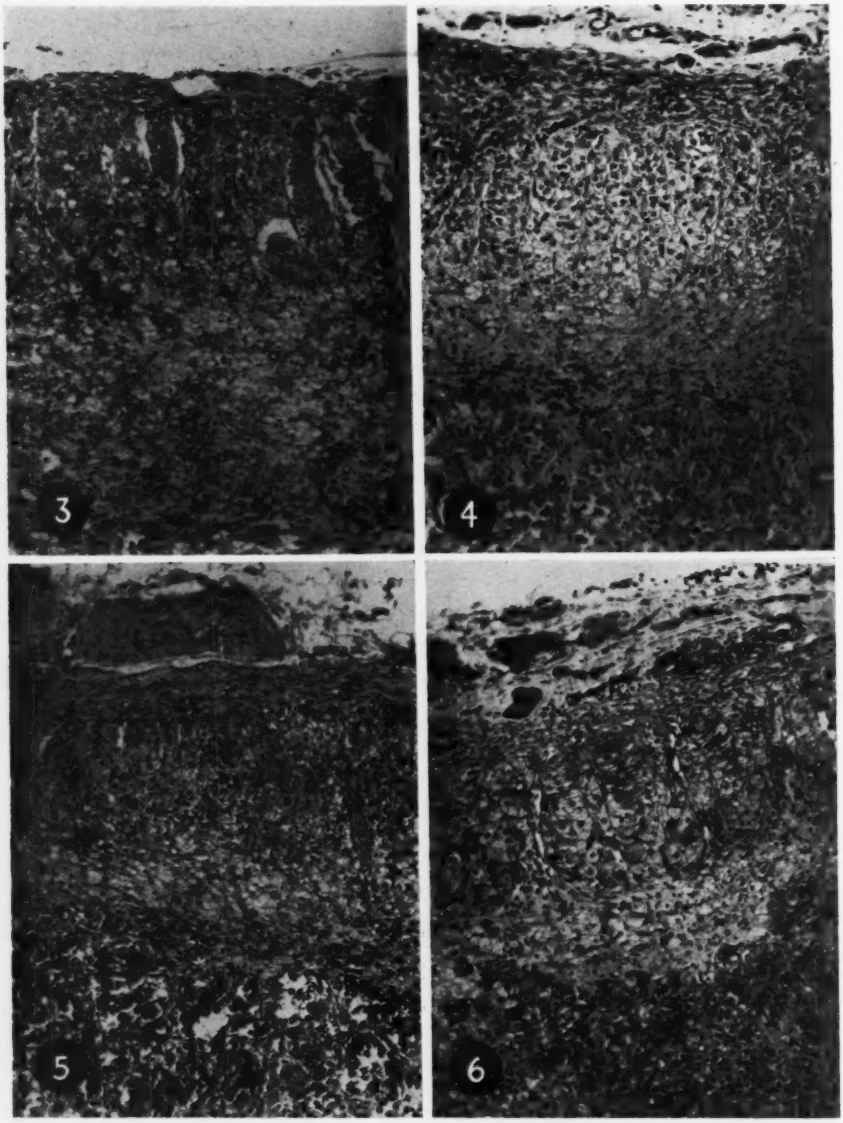

FIG. 3. Degenerative changes in deeper part of adrenal cortex. 23 days treatment with DDD. (×110)

FIG. 4. Gross atrophy of all deeper zones of adrenal cortex, and condensation of their stroma to a fibrous layer. 35 days treatment with DDD. (×110)

At about 10 days the most significant change was an atrophy of the zona reticulata. Many of the cells had disappeared, and the remainder were pressed down so that they lay parallel to the cortico-medullary junction. As a result of this atrophy, the cortex was of only about two-thirds the normal thickness. The remaining cells of the zona reticulata and all those in the deeper half of the zona fasciculata were grossly distended with lipoid, and their nuclei showed a peculiar herring-bone crumpling. There was still a moderate congestion in the deeper cortex, but only in patches.

At about 20 days the gross lipoid vacuolation and nuclear distortion of the cells of the zona fasciculata continued, but there was no remaining congestion (Fig. 3). All the deeper part of the cortical parenchyma had disappeared, its site being represented only by condensed stroma with rather flattened capillaries. As a result the cortex was of only about one half the normal thickness. The zona glomerulosa showed minor changes at this stage: (a) there was sometimes moderate edema between the columns, (b) sometimes the cells showed moderate excess of lipoid, and, as a result of the swelling, had lost their normal palisaded arrangement, but their nuclei were unaffected.

The appearances after 5 weeks' continued treatment with DDD were very striking. The outer quarter of the cortex was almost normal apart from a slight increase of lipoid, but the inner two-thirds or more was represented only by a broad band of condensed stroma whose fibrils appeared swollen and indistinct (Fig. 4). Around this, there was a thin and rather variable layer of grossly vacuolated cells which were full of lipoid (Fig. 5). The nuclei of these cells were rather warped, but not so distorted as in the swollen cells at 10 and 20 days. The cortex was of only about one third the normal thickness.

The 5 dogs which had had 5 weeks' treatment with DDD, and had survived the hypoglycemic phase after the alloxan, were killed at various times from $3\frac{1}{2}$ to 8 days later. During this last period they had received no DDD. The adrenals of these animals showed the same severe atrophy of the cortex as before; the cortex was about one third of the normal thickness. The inner part was represented by condensed stroma, but there were few or no remains of the band of vacuolated cells external to it. The outer

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FIG. 5. Atrophy of deeper part of cortex, but with some remaining lipoid-filled cells beneath the intact outer zone. 35 days treatment with DDD. (×110)

FIG. 6. Healthy outer zone of cortex, projecting into the fibrous layer which represents the deeper zones. 35 days treatment with DDD, followed by one dose of alloxan and no further treatment for 6 days. (×110)

quarter of the cortex was however very different from what it was in the dogs under continuous DDD treatment. There was a well-defined zona glomerulosa with short columns of cells running inwards from it and giving the structural appearance of the outer part of a zona fasciculata. These columns terminated sharply where they met the fibrous tissue remnant of the remainder of the deep cortex (Fig. 6). The cells of this outer cortex had an unusually solid cytoplasm which was strongly eosinophil and contained practically no lipoid; the little that was present being in very fine droplets. The nuclei of these cells were quite healthy.

Cellular infiltration

In dogs dying at 3 days after beginning the DDD treatment, there was a very extensive cellular infiltration in the septa of the zona glomerulosa. The cells were mainly of histiocytic type, with an admixture of large monocytes and some lymphocytic cells.

During the next 2 or 3 days this cellular infiltration appeared to migrate deeper into the cortex, so that it was very prominent in the outer part of the zona fasciculata, and it spread in occasional foci down as far as the zona reticulata. The proportion of lymphocytic cells was higher.

At about 10 days the infiltration was more diffuse but was mainly confined to the deeper part of the zona fasciculata and the atrophying zona reticulata. It was now predominantly lymphocytic, and there was some development of plasma cells.

At about 20 days most of the cellular infiltration had gone, and was represented mainly by scattered lymphocytes and plasma cells in the condensed stroma of the original deeper layers of the cortex. Some of the endothelial cells of capillaries in this region had swollen nuclei. Occasional foci of monocytic infiltration were still present in the outer part of the zona reticulata.

At 5 weeks, there were only very rare lymphocytes or plasma cells in the fibrous tissue zone.

Throughout the whole series there were never any appearances which could be interpreted as myeloid metaplasia (Selye and Stone, 1950).

Medulla

The adrenal medulla did not show any atrophy or necrosis. The cells had an unusually solid cytoplasm containing numerous very fine neutrophil granules; this was in marked contrast to the lacy and loosely granular appearance of the cytoplasm of medullary cells in normal animals. The change was seen as early as 3 days after beginning DDD treatment, and was present in all but two of the treated animals.

Pancreas

There were no recognisable changes in the islets of the dogs treated with DDD alone.

The two DDD-treated dogs, which died 24 to 34 hours after being given the alloxan, showed the characteristic necrosis of the beta cells of the islets (Dunn *et al.*, 1943), similar to the appearances seen in the control dog which died 30 hours after alloxan.

The DDD-treated dogs which were killed at $3\frac{1}{2}$ to 8 days after alloxan had very few islets, and these consisted almost entirely of alpha cells with very occasional degranulated beta cells. Similar appearances were seen in the control dogs which were killed 9 and 13 days after alloxan.

Pituitary

In the animals which died during the period of administration of the DDD, the eosinophil and basophil cells of the anterior lobe were unusually weakly staining. In general there seemed to be a low proportion of eosinophil cells.

In the five animals which were killed $3\frac{1}{2}$ to 8 days after giving alloxan and after ceasing DDD treatment, the eosinophil and basophil cells all gave strong staining reactions. Both types of cell were present in large numbers, and there were few chromophobe cells.

Other organs

No lesions were seen in the kidneys of dogs treated with DDD alone. The DDD-treated dogs which died or were killed at 1 to 6 days after alloxan usually showed necrosis of the epithelium of a few proximal convoluted tubules, with regeneration corresponding to the period of survival.

The liver cells occasionally had a slight degree of fatty change in animals treated with DDD for over 12 days.

No changes were observed in the myocardium or the gonads.

DISCUSSION

Histological changes

Adrenal

The atrophy and final complete loss of the deeper two-thirds of the cortex appears to progress from within outwards. The cells become grossly distended by lipoid, their nuclei become warped, and then the cells disappear. The final result is that only the stroma remains, and this condenses into a flattened layer between the medulla and the remaining

cortex. The remaining cortex consists almost entirely of zona glomerulosa with possibly some of the outer part of the zona fasciculata (Figs. 7 and 8).

The exact cause of this process is not clear. It is known that DDD is taken up by the cells of the adrenal cortex and remains there for a long time (Finnegan, Haag and Larson, 1949), and it might therefore be considered that the pathological lesions were due to a direct toxic effect of the drug. As against this, if the apparent early stages of recovery in the

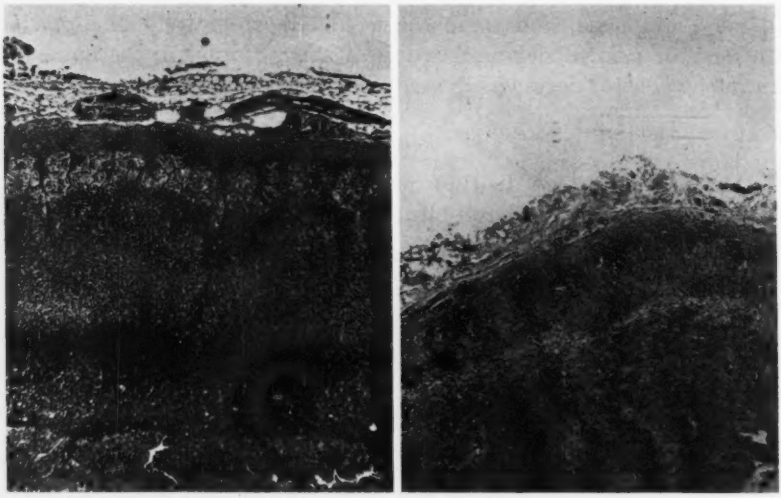

FIG. 7. Left. Adrenal cortex of normal dog. (×30)
FIG. 8. Right. Atrophied adrenal cortex of dog treated for 35 days with DDD. (×30)

remaining cortex in the 5 dogs which had had no DDD for 3½ to 8 days were in fact related to this short period without DDD treatment, it is possible that the significant factor in producing the atrophy is not the DDD in the adrenal but a continuing level of DDD in the blood.

It must however be pointed out that the more healthy appearance of the cells of the outer cortex in these 5 animals is not clearly decided by the present material. The difficulty is that these dogs had been subjected to three new factors: (a) the stimulus of the alloxan, (b) the cessation of DDD treatment for the last 3½ to 8 days of life, (c) the potassium tolerance test. From other experimental material of the present type, but in which the potassium tolerance test was omitted, it is clear that the cytological change in the adrenal cortex is not due to the potassium. The influence of the other two factors remains for further study.

The DDD may operate by blocking the action of adrenocorticotrophic hormone in some way, as has been suggested elsewhere (Nichols and

Gardner, 1951; Nichols and Davis, 1953). This explanation is supported by the striking similarity of the adrenal glands in DDD-treated dogs to those in hypophysectomised animals and in human cases of hypopituitarism. It is of interest that DDD does not appear to affect the human adrenal (Sheehan *et al.*, 1953).

The course of the cellular infiltration in the adrenal cortex is of an unusual type. It might perhaps be argued that the heavy cellular infiltration in the dogs which died early was an indication of some pre-existing disease in these animals, and that this disease had made the dogs very susceptible to the toxic effects of DDD. This explanation appears incorrect for three reasons: (a) the dogs which were killed at this stage had similar cellular infiltration of the adrenal cortex but had no recognisable illness nor any obvious toxic effects from the DDD, (b) the infiltration followed a regular pattern of development depending on the duration of the DDD treatment, (c) the same cellular infiltration has been observed after DDD treatment by workers elsewhere.

The change in the adrenal medulla involves the cytoplasmic structure of the cells, and is presumably an indication of some alteration of function. This could well be secondary to the gross changes in the cortex.

Pancreas

The alloxan produced the same degree of destructive effect on the beta cells of the DDD-treated dogs as it did in the control dogs. The DDD does not appear to have protected the islets in any way from the alloxan. The differences between the biochemical changes in the two groups of dogs can thus not be ascribed to any difference in the degree of damage to the islets.

Other organs

In the animals killed a few days after alloxan and after the cessation of treatment with DDD, the changes in the staining reactions of the cells in the pituitary are similar to the changes in the adrenal cortex at that stage and are presumably related.

The necrosis of renal tubules after alloxan is a standard effect of this toxin. It was not sufficiently severe in the present cases to produce any serious renal insufficiency.

Biochemical changes

The various phases of the blood sugar curve after alloxan will be considered firstly as they occur in the normal dog and secondly with regard to the effect of previous treatment with DDD. In view of the loss of all the deeper zones of the adrenal cortex in the DDD-treated dogs, and the fact that the islets are damaged equally in both groups of dogs, it appears

reasonable to conclude that the difference in biochemical response is due to the adrenal atrophy produced by the DDD.

Initial fall of blood sugar

The phase of slight hypoglycemia during the first hour after alloxan is usually not recognised, because most observers do not study the blood sugar during the first hour. It has however been recorded in dogs by Shipley and Beyer (1947). More recently, Wrenshall, Collins-Williams and Best (1950) have shown that it is of constant occurrence if, before administration of the alloxan, the dogs have either been maintained on a low carbohydrate and high fat diet, or have been treated with insulin. We know of no explanation.

Effect of DDD. This phase was observed in 6 of the 10 animals used in the experiment; there was no obvious difference between the DDD-treated dogs and the controls.

Primary phase of hyperglycemia

This hyperglycemic phase after alloxan is of regular occurrence in all normal animals. The main clue to its etiology is that it is known to be abolished by complete removal of the adrenals in the rat (Kirschbaum *et al.*, 1945) and in the rabbit (Iverson, 1947).

(a) These authors, and also Duff (1945), accept that this effect of adrenalectomy is due to the loss of the adrenal cortex. The explanation is supported by the fact that hypophysectomy in the dog (de Oya and Covián, 1946) or in the rabbit (Iverson, 1947) also prevents the development of the hyperglycemia, presumably by causing loss of function of the deeper layers of the adrenal cortex, though it is of course possible that the loss of pituitary hormones may also have a direct effect on carbohydrate metabolism.

(b) An alternative view is that adrenalectomy abolishes the primary phase of hyperglycemia because it deprives the animal of its adrenal medulla (Goldner and Gomori, 1944; Brown *et al.*, 1945). This does not explain how hypophysectomy, which does not affect the medulla, nevertheless abolishes the hyperglycemia. In addition, it has been shown by Kirschbaum *et al.* (1945) and Houssay *et al.* (1946) that the animal with a normal adrenal cortex but no adrenal medulla gives a normal hyperglycemic response to alloxan. Further evidence that the hyperglycemia is not due to epinephrine is that Regitine, a drug which blocks the hyperglycemic effects of that hormone (Trapold *et al.*, 1950), does not prevent the primary hyperglycemic phase after alloxan (Nichols and Little, 1952).

(c) Hughes, Ware and Young (1944), testing the hypothesis that the initial hyperglycemia is due to epinephrine and the subsequent hypogly-

cemia to release of insulin, injected into rabbits a mixture of these hormones in the amounts that are normally present in the pancreas and adrenal medulla. This produced an initial hyperglycemia followed by a phase of hypoglycemia; the course of the blood sugar being very similar to that produced by alloxan. In interpreting the results, the possible effect of the injected epinephrine on the pituitary-adrenal system must be borne in mind. It can not be concluded from the experiment that the rise of blood sugar which follows the injection of alloxan is necessarily due to the release of epinephrine; the other factors which can cause hyperglycemia are not excluded.

(d) Denervation of the adrenal does not affect the initial hyperglycemia after alloxan in the dog (Houssay *et al.*, 1945), in the cat (Perlata, 1945), or in the rat (Shipley and Beyer, 1947). This observation can, however, be interpreted in many ways, and it can not be accepted as proof that the medulla is not responsible for the hyperglycemia.

Effect of DDD. This phase of hyperglycemia is almost completely absent in dogs whose adrenal cortex has been made atrophic by DDD. If it be accepted that the association indicates a causal relationship, it may be concluded that the hyperglycemic phase after alloxan in the control animals is mediated by the deeper zones of the adrenal cortex. This interpretation is in accordance with the evidence in the literature as to the effect of hypophysectomy.

Phase of severe hypoglycemia

Three hypotheses have been put forward to account for this phase: (a) The hypoglycemia is produced by the liver, probably because of some direct effect of alloxan on that organ (Houssay *et al.*, 1945). When the liver has been poisoned with phosphorus, this hypoglycemic phase does not occur (Bhattacharya, 1950). (b) After adrenalectomy or hypophysectomy, the hypoglycemic phase occurs earlier and is more severe in the rat (Martinez and Orias, 1945; Lammers and Gaarenstroom, 1950) in the rabbit (Iverson, 1947) and in the dog (Shipley and Beyer, 1947). This may be merely another aspect of the suppression of the primary hyperglycemic phase which results from excision of these endocrine organs. (c) The hypoglycemia has been attributed to the release of insulin from the dead beta cells; this subject has been reviewed by Lukens (1948). An objection to this explanation is that the insulin content of the pancreas in the dog is the same 3 hours after the hypoglycemic phase has begun as it was at the time the alloxan was injected (Wrenshall, 1946, quoted from Wrenshall *et al.*, 1950).

Effect of DDD. The dogs treated with DDD had a very early onset of the hypoglycemia, similar to the course seen after adrenalectomy or

hypophysectomy. The duration of this phase was, however, no longer than in control animals. The factor which produces the hypoglycemia thus appears to be self-limiting and not to be under the control of the adrenal cortex, apart from the fact that it does not come into operation until the primary hyperglycemic phase has passed. The intensity of action of the factor which produces the hypoglycemia can not be judged from the blood sugar levels, because the animals were given glucose continuously at that time. It seems however that the factor did operate more strongly in the DDD-treated animals, because much larger amounts of glucose were required to keep these dogs alive and because, despite intensive administration of glucose, two of the dogs died.

Final phase of hyperglycemia

About 48 hours after the administration of alloxan, normal dogs have become permanently diabetic. The hyperglycemia appears to be dependent on the function of the adrenal cortex, because it is ameliorated by adrenalectomy or hypophysectomy. This effect has been demonstrated in rats (Kirschbaum *et al.*, 1945; Gaarenstroom and de Jongh, 1946; Bailey *et al.*, 1947), in rabbits (Houssay *et al.*, 1945), and in dogs (Houssay, 1947). It was at one time suggested that the reduction of the blood sugar might be due merely to the lowering of food intake as a result of the operation (Burn *et al.*, 1944; Janes *et al.*, 1945), but it is now known that hypophysectomy still ameliorates the diabetes when the animals are force-fed to their pre-operative food intake (Gitter and Retti, 1948). It is of interest that spontaneous diabetes in man can be influenced by hypophysectomy (Chabanier *et al.*, 1936) adrenalectomy (Green *et al.*, 1950) or adrenal atrophy (Laqueur and Harrison, 1951) in the same way as alloxan diabetes in animals.

During the course of alloxan diabetes in several species of animals, various histological changes develop in the adrenal, the anterior lobe of the pituitary, and the thyroid (Hard and Carr, 1944; Kendall *et al.*, 1945; Thomas and Emerson, 1945; Ruben and Yardumian, 1946; Bennett and Koneff, 1946; de Majo, 1947; Desclaux *et al.*, 1948a and b; Soulairac and Desclaux, 1948; Covián, 1949; Eränkö, 1950; Applegarth, 1949). The changes can be interpreted in several ways, but certainly seem to indicate that these glands are involved in the mechanism of continued alloxan diabetes.

Effect of DDD. In the dogs which had been treated with DDD, there was little or no elevation of the blood sugar during the week after the alloxan. It is difficult to escape the conclusion that this was a result of the severe atrophy of the adrenal cortex; practically every beta cell in the islets had been destroyed in these animals just as in the control dogs.

On histological grounds it seemed that, in the dogs which had been given alloxan but no further DDD during the last few days of life, there was possibly some very early regeneration of zona fasciculata from the intact zona glomerulosa. If the animals had been allowed to remain alive longer so that any such regeneration had had time to develop, it is possible that they might have become diabetic; Greep and Dean (1949) have shown that cortical tissue which has regenerated from the zona glomerulosa can secrete glucocorticoids.

Potassium tolerance test

Allers *et al.* (1936) reported that 5 gm. of KCl will cause a fatal crisis in a bilaterally adrenalectomised dog within a few hours. The dogs which had been treated with DDD were given about twice this dose. Their adrenals had an intact zona glomerulosa, and there was no recognisable effect from the administration of the potassium. This is in line with current concepts that the function of the zona glomerulosa is the regulation of electrolyte balance, and that the zona fasciculata is concerned mainly with carbohydrate metabolism. Nevertheless it must be recognised that this division of function is probably not absolute. Brownell *et al.* (1950) have shown that rats which have had the zona fasciculata and reticulata curetted out from the adrenal, leaving an apparently normal zona glomerulosa, suffer from some slight deficiency in electrolyte balance. Furthermore, adrenocorticotrophic hormone, which affects mainly the zona fasciculata but also to a slight degree the zona glomerulosa (O'Donnel *et al.*, 1951), does cause a slight and transitory sodium retention (Sprague *et al.*, 1950). Similarly, hypophysectomy in dogs, which produces total atrophy of the zona reticulata and inner zona fasciculata, does also lead to some atrophy of the zona glomerulosa (Lane and de Bodo, 1952).

SUMMARY

A group of dogs were given DDD orally for 5 weeks. This treatment produced atrophy and disappearance of the inner two-thirds of the adrenal cortex, leaving little more than the zona glomerulosa intact. Alloxan was then injected in a dose which destroyed almost all the beta cells of the islets. The phase of primary hyperglycemia was short and of minor degree, but the phase of subsequent hypoglycemia occurred early and was unusually severe. During the following few days the dogs had blood sugars little if any higher than normal. Finally the dogs were subjected to a potassium tolerance test without ill effect.

The suppression of the initial hyperglycemia and of the diabetes following alloxan is interpreted as due to the loss of the zona fasciculata and the zona reticulata of the adrenal cortex in these DDD-treated dogs. The

satisfactory electrolyte control during the potassium tolerance test is considered to indicate good function by the surviving zona glomerulosa.

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THE ANTAGONISTIC EFFECTS OF ESTROGEN AND PROGESTERONE ON THE STAIRCASE PHENOMENON IN UTERINE MUSCLE

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THE "staircase" phenomenon is well known to be exhibited by cardiac muscle, but has not been established as characteristic of smooth muscle. As the present paper will demonstrate, the staircase phenomenon readily occurs in experiments on rabbit uterine muscle. Its occurrence has a certain uniformity if the muscle is taken from animals that are in similar condition with respect to the ovarian hormones. If, however, the hormonal status of the animal is varied, the form of the staircase is altered in a way highly characteristic of the dominant ovarian hormone.

Unlike earlier studies on the staircase phenomenon our experiments have utilized isometric rather than isotonic recording, inasmuch as the former method has been shown to yield more accurate quantitative results (Hajdu and Szent-Györgyi, 1952). The contractions were not only elicited, as in previous studies, by a series of stimuli at constant frequency after a period of rest, but also by varying the frequency of stimulation in a steady state (Dale, 1932).

METHODS

Fifty mature virgin female New Zealand White rabbits weighing 3 to 4 kg. each were used. Forty-seven of these were subjected to bilateral ovariectomy under anesthesia. Twenty-one days after ovariectomy 8 were given 50 gamma per day of natural estrogen[2] injected in oil intramuscularly for 7 days. Hereafter the term "estrogen-treated" refers to this group. Four others received a similar 7-day course of estrogen treatment followed by injection of progesterone in oil intramuscularly, 1 mg. per day for 3 days. This group will be called "progesterone-treated." Seven animals received no treatment following the 21 days allowed for establishment of the effects of castration. We shall refer to these animals as "castrates." The term "estrogen-withdrawn" designates 3 animals which received a standard estrogen treatment beginning 21 days after castration, but after the last injection of estrogen were kept without hormone treatment for 4 days. The other ovariectomized animals in this series of experiments received different amounts of

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[2] The estrogen used was Amniotin (mixed estrogens, potency expressed in estrone equivalents). Thanks are due to E. R. Squibb and Company for the gift of a supply of the hormone.

ovarian hormones for different lengths of time and also the proportion of the two hormones was varied. No group characterization of these animals is possible, therefore, at present and they will only be mentioned in the discussion. Two animals were used in normal early pregnancy (5 and 7 days) and 1 in natural estrus (determined by successful mating).

Twenty-four hours after the last injection, under Nembutal anesthesia (40 mg. per kg. body weight intravenously) the uterine horns were rapidly removed and placed in continuously oxygenated Krebs' solution at 0° C. for 10 minutes. A strip 25 mm. long and about 12 mm. wide was then cut from the uterus and placed between 2 platinum hooks in a vertical glass tube filled with Krebs' solution. By immersion in a water bath the tube was kept at a temperature of 37.5° C. Through an inlet at the bottom of the tube a mixture of 95% oxygen and 5% CO_2 was bubbled through the Krebs' solution. The oxygen concentration in the Krebs' solution was about 2.0 volumes per cent and the pH remained constant at 7.4.

The lower hook being fixed, the upper one was attached by a silk thread to the short arm of a conventional isometric tension recorder in which the lever is suspended on a flat torsion spring of brass. A writing point on the long arm of the lever records the shortening of the muscle when it contracts, but since the ratio of the long to the short arm is 37 to 1 the shortening is very slight ($<6\%$ of the resting length of the muscle strip). The contraction is, therefore, practically isometric, the torsion of the lever registering the tension produced. Electrical stimuli of 5 seconds duration, 24 volts, 60-cycle A.C., were sent via the platinum hooks by an automatic timer at intervals of $\frac{1}{2}$, 1, 2, and 4 minutes. In a series of previous experiments this type of stimulation was found to be optimal for rabbit uterine strips, securing a uniform pattern for any desirable length of time. Alterations in the voltage ($\pm 25\%$) or in the duration resulted in submaximal tension. In all the experiments referred to in this paper all the experimental conditions were kept as constant as possible.

The observations were begun by removing the uterine strip from the cold Krebs' solution and placing it in the apparatus in oxygenated Krebs' solution at 37.5° C., where it was left for 10 minutes before beginning electrical stimulation.

RESULTS

As shown in Figure 1 A, as stimulation is begun at 1-minute frequency, the muscle strip taken from an estrogen-treated castrate exhibits a gradual increase in tension, the maximum being reached only after about 15 successive stimuli. This behavior is similar to that previously known in cardiac muscle. The maximum tension, once attained, will remain quite constant for several hours if the frequency of stimulation and the experimental conditions (temperature, pH, ionic strength, energy stores, etc.) are unaltered. This gradual increase in tension after stimulation begins is the staircase phenomenon. If the electrical stimulation is discontinued and then resumed, the staircase reoccurs. The uterine strip taken from an animal under natural estrogen domination, i.e., in natural estrus, exhibits similar behavior with respect to the staircase.

If the uterine strip is taken from a progesterone-treated animal, the form of the staircase will be quite opposite to that seen previously, although

the experimental conditions are unchanged. As seen in Figure 1 B, stimulation at 1-minute frequency following a 10-minute resting period results in a gradual decrease in tension until after some 15 stimuli a steady state is reached, the tension remaining unaltered thenceforward. This peculiar behavior has not been reported previously and is characteristic of the effect of progesterone upon the myometrium. This phenomenon will be called hereafter "negative staircase." Uterine strips from rabbits under natural progesterone domination, i.e., in early pregnancy, exhibit similar behavior with respect to negative staircase. If the ovaries are removed from the mature rabbit, the production and effects of the ovarian hormones cease and three weeks later obvious morphological, chemical, and func-

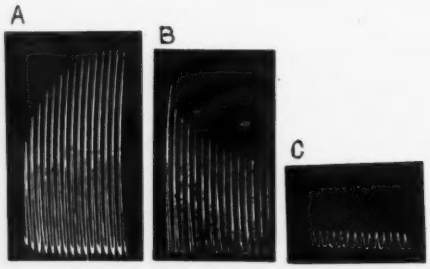

FIG. 1. Isometric study of the staircase phenomenon of rabbit uterine strips *in vitro* stimulated with constant frequency. *A*. Mature virgin female rabbits 21 days after ovariectomy, treated for 7 days with 50 gamma per day estrogen in oil intramuscularly. Experiment performed 24 hours after the last injection of estrogen. Note the gradual increase in tension with successive stimuli. *B*. The same as A except that after estrogen treatment the animal was injected intramuscularly for 3 days with 1 mg. of progesterone in oil per day. Note the gradual decrease in tension, in contrast with A. *C*. The same as A and B, but without hormone treatment. No change in tension. All graphs represent typical behavior within a group of several experiments. Rate, 1 contraction per minute.

tional alterations are well established. Changes in the concentration of contractile proteins after ovariectomy have previously been described (Csapo, 1950). Changes in the concentration of high-energy phosphates and in the maximum tension per unit cross section area have been observed in our laboratory and will be reported elsewhere. It may be mentioned, however, at this point that uterine strips taken three weeks after castration exhibit no staircase of any kind, as shown in Figure 1 C. The great reduction in maximum tension is mainly due, according to our observations, to changes in the concentration of the contractile proteins. With such small tensions the measurements are less accurate. It is quite clear, however, that the tension developed after previous rest is unaltered throughout a series of successive contractions.

As pointed out, the staircase can be studied more accurately if instead of varying the time of rest between two periods of stimulation with identical frequencies, the frequency of electrical stimulation is varied systematically after a steady state of maximum tension has been reached.

The graphs of Figure 2 were obtained in the following way. As in the experiments shown in Figure 1, the muscle strips were first stimulated at the rate of 1 per minute. After maximum tension was obtained with this frequency, the rate was changed to 1 every half minute. About 15 stimuli with this frequency were necessary thereafter to reach a steady state and keep the tension constant. Then 7 contractions were recorded at $\frac{1}{2}$-minute frequency, 4 at 1-minute, 3 at 2-minute, and 2 at 4-minute frequency.

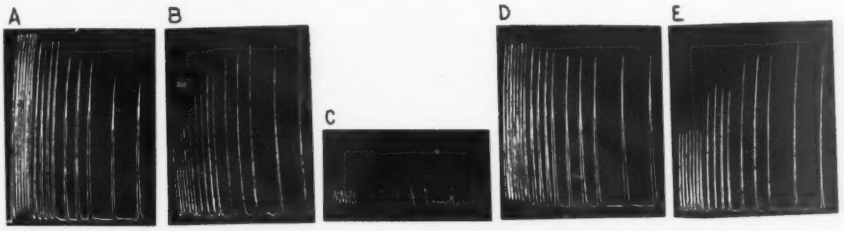

FIG. 2. Detailed isometric study of the staircase phenomenon of rabbit uterine strips *in vitro*, by varying the frequency of electrical stimulation in a steady state. *A*. Estrogen-treated castrates (as in Figure 1 A). Note the decrease in tension with decreasing frequency of electrical stimulation. *B*. Estrogen- plus progesterone-treated castrates (as in Figure 1 B). Note increase in tension as frequency decreases. Speed of drum as in Figure 1; stimulation intervals $\frac{1}{2}$ to 4 min. *C*. Castrate without treatment (as in Figure 1 C). Note no change in tension with changing frequency. *D*. Rabbit in natural estrus. *E*. Animal pregnant 7 days. All graphs represent typical behavior within a group of several experiments.

As Graph A demonstrates, the uterine strips from estrogen-treated castrates exhibit a decrease in maximum tension as the frequency of electrical stimulation decreases. This behavior is a more useful quantitative expression of the same phenomenon shown in Figure 1 A and will also be called staircase.

On the contrary, uterine strips from progesterone-treated rabbits exhibit an increase in tension with decrease in frequency of electrical stimulation, as shown in Graph B. This phenomenon will be called hereafter negative staircase. As expected from the experiments shown in Figure 1, the uterine strip from a castrate without hormone treatment exhibits no change in tension with varying frequency of stimulation, as shown in Graph C. In view of this striking dependence of the staircase phenomenon upon endocrine conditioning of the uterus, as seen experi-

mentally in castrate animals treated with the ovarian hormones, it is obviously important to know whether uterine muscle exhibits the same behavior in the varying hormone states associated with natural reproductive conditions, i.e., estrus (estrogen domination) and pregnancy (progesterone domination). The graphs of Figure 3 summarize the results of an investigation of this question as well as of the experiments on hormone-treated castrates. As shown in Graph A, natural estrogen domination results in a staircase similar to that of the estrogen-treated castrates

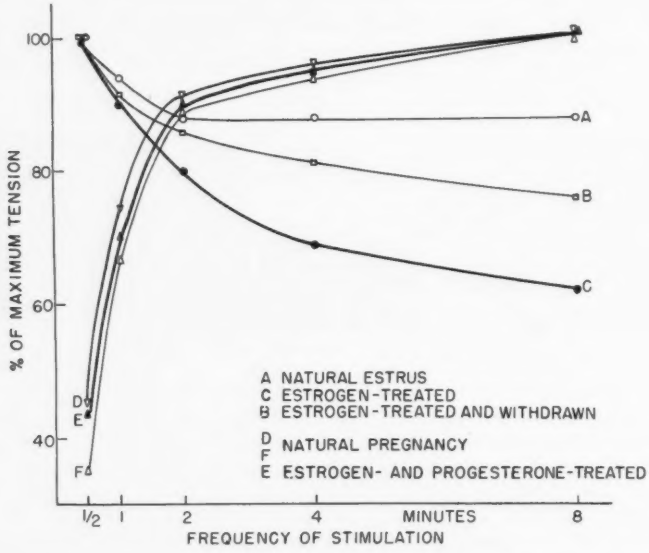

FIG. 3. Summary of experiments with respect to the slope of staircase and negative staircase from estrogen- and progesterone-dominated rabbit uteri. *A*. Animal in natural estrus (single case). *B*. Average of 3 cases. Rabbits as C, but 4 days after the last estrogen injection. *C*. Average of 7 cases. Estrogen-treated castrates. Individual variation within each group represented in this figure is less than ± 10 per cent. *D*. Seven-day pregnant (single case). *E*. Average of 4 cases. Animals as B, but estrogen treatment followed by 3-day progesterone treatment. *F*. Five-day pregnant (single case).

(Graph C). It can also be seen, in Graphs D and F, that natural progesterone domination results in a negative staircase similar to that of progesterone-treated castrates (Graph E).

If one plots the tension (as per cent of maximum tension attained) against the frequency of stimulation, from a series of ovariectomized animals treated identically with estrogen and with estrogen plus progesterone respectively, the slope of the staircase and the negative staircase (pro-

duced by the respective hormone treatments) can be ascertained and the variations of their slope in individual animals can be observed.

Curve C of Figure 3 represents the average slope of staircases obtained from identical experiments with uterine strips of the 7 estrogen-treated castrates. The individual variation of the maximum tension at each frequency was less than $\pm 10\%$ here and thereafter. Curve A shows the slope of the staircase of a uterine strip from a rabbit in natural estrus. The slope is less steep than that of Curve C. Curve B represents the average slope of the staircases of 3 rabbits treated like those represented by Curve

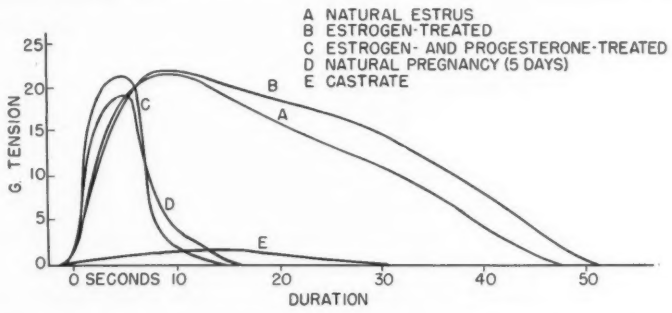

FIG. 4. Duration of single contraction-relaxation cycles of rabbit uterine strips under estrogen and progesterone domination. The duration curves are superimposed upon each other from the original records. Each curve shows the typical behavior of the represented group. Stimulus at optimal frequency for each hormonal status.

C, except that the administration of estrogen was followed by a period of 4 days without treatment. The average slope of the staircase of this group lies between A and C. Curve E shows the average slope of the negative staircases of 4 rabbit uterine strips treated with estrogen plus progesterone. This curve is almost identical with Curves D (pregnant, 7 days) and F (pregnant, 5 days).

The general similarity of Curves A, B, and C (estrogen-dominated) and the almost perfect identity of D, E, and F (progesterone-dominated) clearly demonstrate that the hormone-dependence of the staircase phenomenon of rabbit's uterine muscle is the same whether the hormones are produced by the animal's own ovaries or received by injection. The opposite slope of the staircase under estrogen and progesterone domination respectively is also strikingly shown.

When the duration of a single contraction-relaxation cycle at optimum frequency of stimulation (for maximum tension) is recorded on a fast drum, obvious differences become apparent between estrogen- and progesterone-dominated uteri (Figure 4). Whereas the estrogen-dominated strips

(A and B) have a duration of about 50 seconds, the progesterone-dominated strips (C and D) have a duration of about 15 seconds, and the uteri of castrates (E) a duration of about 30 seconds. Thus one can see that progesterone reduces the duration of the contraction-relaxation cycle of an estrogen-dominated uterus by about 70 per cent.

The graph also shows that the speed of contraction and also of relaxation of progesterone-dominated uterine strips is faster than in estrogen-dominated uteri. It is easy to see where relaxation begins in the case of progesterone domination (C, D), but quite difficult in the case of estrogen domination (A, B), because the decrease in tension is more gradual. It can be safely said, however, that the estrogen-dominated strip holds its tension at a high level about 5 times as long as does a strip from the progesterone-dominated uterus.

DISCUSSION

The observations that no staircase can be observed with the uterine strips of castrates, that the staircase appears if estrogen is administered to the living animal, and that this effect of estrogen can be completely reversed by progesterone, with resultant negative slope of the staircase, suggest that the staircase is not a property of the uterine muscle, but an expression of the effect of the dominant hormone upon the myometrium. The effect of the two ovarian hormones upon the staircase phenomenon is clearly antagonistic. It follows, therefore, that administration of the two hormones combined in suitable proportions should result in intermediate stages in the slope between extreme staircase and negative staircase. Preliminary experiments of this sort have been performed with success and will be reported when complete.

Not only does the staircase phenomenon demonstrate the antagonism between estrogen and progesterone, but so does the duration of a single contraction cycle. One hundred gamma of progesterone per day as before significantly decreases the duration, but a daily dose of only 500 gamma results in typical behavior of progesterone domination. Taking the castrate as a base line, estrogen increases the duration, whereas progesterone greatly decreases it, the maximum tension remaining unaltered. The observation of Hajdu and Szent-Györgyi (1952) made of the frog heart that maximum tension and duration go parallel, therefore, does not hold for the uterus as a rule.

At this stage of our work, it is not possible to draw conclusions about the nature of the effect of the ovarian hormones upon the myometrium. In view, however, of the conclusion of Hajdu and Szent-Györgyi (1952) that the intracellular potassium concentration and the consequent differences in the Na/K ratios inside and outside of the cell determine the behavior

of the staircase in cardiac muscle, it is tempting to suppose that the ovarian hormones affect the myometrium through their influence on the potassium balance of the uterine cell. Experiments aimed at influencing the potassium concentration of uterine strips by indirect methods have been begun in our laboratory with suggestive, but as yet inconclusive, results.

SUMMARY

The occurrence of a staircase phenomenon in uterine muscle *in vitro* has been demonstrated. The development and slope of the staircase is determined by the ovarian hormones. The staircase is elicited by successive electrical stiumulations after rest, or by varying the frequency of stimulation after a steady state of muscle response is established.

After ovariectomy no staircase occurs. Uterine strips taken from rabbits in natural estrus or from castrates treated with estrogen and studied 24 or 96 hours after the last injection exhibit a typical staircase phenomenon. Strips taken from rabbits in early pregnancy or from castrates treated with progesterone following a course of estrogen exhibit a negative staircase, i.e., increase in tension with decreasing frequency of electrical stimulation.

The ovarian hormones were also found to alter the duration of a single contraction-relaxation cycle of a uterine strip *in vitro*. Taking the castrate as a base line estrogen increases whereas progesterone decreases the duration of a single contraction cycle.

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NUCLEIC ACID PATTERNS IN RAT LIVER FOLLOWING HYPOPHYSECTOMY AND GROWTH HORMONE ADMINISTRATION[1]

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INTRODUCTION

THE administration of purified pituitary growth hormone to either hypophysectomized or intact rats results in nitrogen retention and increased protein synthesis. However, the mechanisms involved are unknown. Recently, several investigators have described high concentrations of ribosenucleic acid (RNA) in cells actively engaged in protein synthesis (Brachet, 1950; Caspersson, 1950; Davidson, 1950) and increases of this nucleic acid in cells which have an accelerated rate of protein synthesis. This constitutes circumstantial evidence that the concentration of RNA in cells may be related to their protein anabolic activity.

That growth hormone may exert its protein anabolic effect by a mechanism involving nucleic acid metabolism is suggested by at least two recent studies. Geschwind *et al.* (1950) have shown that hypophysectomy results in a lowering of the RNA concentration of rat liver, and that growth hormone treatment of hypophysectomized rats restores the liver RNA concentration to normal. Further, Kalter *et al.* (1950) have described enhancement of the growth rate of influenza virus in mice treated with purified pituitary growth hormone. Since influenza virus contains significant amounts of nucleic acids (Knight, 1947) this observation is consistent with the hypothesis that growth hormone may affect nucleic acid metabolism.

The recent development of microspectrophotometric methods for estimating the nucleic acid and protein content of individual cell nuclei as well as cytoplasm (Pollister and Ris, 1947; Pollister, 1949; Swift, 1950) has made it possible to explore the effect of hypophysectomy and growth hormone replacement therapy on the constituent parts of single cells. In the present study observations of this type have been made on rat liver tissue

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and have been correlated with analytical chemical determinations of nucleic acids done on representative samples of the same livers.

METHODS

Male rats of the Sprague Dawley strain were obtained from the Hormone Assay Laboratory, Chicago, Ill. All animals were maintained on a diet of canned dog food and were allowed water *ad libitum*. Four days postoperatively the animals were divided into three groups. One group, intact controls, was given daily intramuscular (I.M.) injections of 0.5 ml. of 0.85 per cent saline for 7 days. One group of hypophysectomized rats received 0.5 ml. of 0.85 per cent saline I.M. daily for 7 days and one group of hypophysectomized rats received 0.5 mg. of growth hormone[2] (Armour & Co. Lot No. 22 KR1)

TABLE 1

Absorption measurement	Wavelength	Wavelength isolation	Lamp	Optical system
Ultraviolet	254 mμ	Cooke double prism quartz monochromator	Hanovia Sc2537 Hg vapor discharge	Cooke quartz cond. N. A. 1.2; 2 mm. glyc. imm. obj. N. A. 1.2; 5×ocular
Feulgen	560 mμ	Farrand interference filter	AH4 Hg arc	Leitz condensor N. A. 1.2; 2 mm. objective N. A. 1.2; 10×ocular Image focused on photosensitive element by means of a convex lens.
Methyl Green	661 mμ	Farrand interference filter	Zirconium concentrated arc	
Azure A	580 mμ	Farrand interference filter	Zirconium concentrated arc	
Millon	365 mμ	Corning glass filter (7-83)	AH4 Hg arc	

I.M. daily for 7 days. All animals were weighed daily. On the day following the last injection the rats were sacrificed and the livers were rapidly removed. A sample of approximately 500 mg. was weighed immediately and placed in a test tube in the freezer compartment of a refrigerator. Nucleic acids were extracted from this sample by the method of Schmidt and Thannhauser (1945) and analyzed for phosphorus by the method of Fiske and Subbarow (1925). Another 500–750 mg. sample was taken for fat analysis (Payne, 1949). For cytological spectrophotometric analyses of protein and nucleic acids, pieces of liver from the same animals were removed immediately and fixed overnight in Carnoy's acetic alcohol (1:3), embedded in paraffin, and sectioned at 3 μ and 10 μ. The apparatus for making absorption measurements has been described by Pollister and Moses (1949). This was used in conjunction with a Photovolt electronic multiplier photometer (model 512 was used for measurement of ultraviolet absorption, while model 520M with the 21c search unit was used for all other absorption measurements). Table 1 indicates wavelengths used in the various absorption studies herein reported, together with the method of isolation from the light source and optical system used.

[2] The purified growth hormone was generously supplied by Mr. Irby Bunding of the Endocrine Research Division of Armour & Co.

Ultraviolet absorption studies

3 μ sections mounted on quartz slides were used for measurements of ultraviolet absorption. Nuclei presenting a round cross-sectional area were measured, as well as cores of cytoplasm 4.4 μ in diameter. In each case the nuclei measured were not whole. Only those which had been cut across both upper and lower poles were chosen (since the diameter of nuclei measured ranged from 6.2 μ to 7.4 μ, interference by cytoplasmic absorption is negligible in a 3 μ section).

The cytochemical ultraviolet procedure has been described by Pollister and Ris (1947) and by Di Stefano (1948). In order to determine the absorption due to nucleic acids, the non-specific absorption, or light loss not due to nucleic acid, was first measured. This value was then subtracted from the total light loss as measured in the test slide. By this procedure total nucleic acids are measured since ultraviolet absorption does not differentiate between RNA and DNA.

DNA Studies

For determination of DNA by means of the Feulgen reaction, sections were hydrolyzed in N HCl at 60° C. for 12 min. (Di Stefano, 1948) and stained with the Schiff reagent prepared according to the directions published by Stowell (1945). DNA studies were also made by means of basic staining with methyl green. This procedure, as described by Pollister and Leuchtenberger (1949), stains "polymerized" DNA. With these staining techniques 10 μ sections were used for the spectrophotometric determinations of DNA. Since, with these procedures, there is no staining of cytoplasm, whole, spherical nuclei having a radius of from 3.1 μ to 3.7 μ were measured.

RNA Studies: (Basic staining with ribonuclease digestion)

10 mg. of crystalline ribonuclease (Armour & Co. Lot. No. 9044X) were boiled for 5 min. in 1 ml. of a saturated solution of ammonium sulfate in order to remove any traces of proteolytic activity (McDonald, 1948) and then made up to 50 ml. in distilled water (pH adjusted to 6 with NaOH). Sections to be digested were placed in this enzyme solution at 45° C. for 3 hours, while control sections were placed for the same period of time and at the same temperature in a solution of ammonium sulfate at a concentration equal to that in the enzyme solution.

Both control and digested sections were then stained with Azur A (Pollister, 1950) a procedure which, according to Flax and Pollister (1949) stains both types of nucleic acid. The RNA value was then computed by subtracting the amount of dye bound after ribonuclease digestion from the value found in control sections.

Spectrophotometric determinations of bound dye in these experiments were made under the same conditions as those of ultra-violet absorption. In 3 μ sections round nuclei of the same size used in other experiments, as well as cytoplasmic cores 2.9 μ in diameter, were chosen for measurement.

Protein Studies

Photometric measurements of the color produced by the cytochemical application of the trichloracetic acid Millon reaction described by Pollister and Ris (1947) were made of nuclei and cytoplasmic cores 2.9 μ in diameter. For the reasons given in the section on ultraviolet absorption studies, round nuclei ranging in diameter from 6.2 μ to 7.4 μ, which in a 3 μ section had been cut across the upper and lower poles were selected for this study.

RESULTS

The results of chemical determinations of liver nucleic acids following hypophysectomy and the effect of growth hormone are given in Table 2. Both experimental values and RNA values recalculated on the basis of a

TABLE 2. LIVER NUCLEIC ACIDS

Experiment	No. of observations	Nucleic acid phosphorus mg./100 gms. liver (fat free wet wt.)					
		DNA-P	P[1]	RNA-P	P	RNA-P Calculated from DNA constant at control level	P
Controls (saline)[2]	14	22.1±0.55*	<0.01	109.3±4.84	>0.1	109.3±4.84	<0.01
Hypophysectomized (saline)[3]	14	27.9±1.45	<0.05 >0.02	109.1±2.60	>.05	78.8±6.15	<0.01
Hypophysectomized + Growth Hormone[4]	10	24.2±0.79		116.4±3.24		104.4±4.01	

[1] P = Probability from Fischer's Table "t".
[2] Controls (same age and sex as hypox) 0.5 ml. of 0.85% saline I.M. daily, 7 days.
[3] 0.5 ml. of 0.85% saline I.M. daily, 7 days.
[4] 0.5 mg. of Growth Hormone (Armour & Co. Lot. No. 22 KR1) I.M. daily, 7 days.

* S. E. computed by: $S.D. = \sqrt{\frac{\Sigma x^2 - (Mx \Sigma x)}{n-1}}$; $S.E. = \frac{S.D.}{\sqrt{n}}$

constant DNA are given. The validity of this recalculation will be apparent by reference to Table 3 which represents the cytochemical data establishing the constancy of DNA per nucleus. Hypophysectomy caused a significant decrease in RNA, a decrease which was not seen when hypophysectomized animals were treated with growth hormone. These data are in agreement with those obtained by Geschwind *et al.* (1950).

The nuclear and cytoplasmic constituents separately determined by

TABLE 3

Class	Animal	DNA* (Feulgen)	RNA		Protein (Million)	
			Nucleus* (Azure A + Ribonuclease)	Cytoplasm† (E_{284})	Nucleus*	Cytoplasm† (E_{45})
Controls	257	14.9±0.3	1.50±0.14	0.199±0.006	4.81±0.22	0.152±0.003
	258	15.6±0.3	1.45±0.16	0.199±0.006	5.28±0.18	0.175±0.006
	259	16.0±0.3	1.19±0.08	0.190±0.006	5.35±0.16	0.157±0.006
	Mean	15.5±0.2	1.38±0.07	0.196±0.003	5.15±0.11	0.161±0.003
Hypophysectomized	233	15.5±0.3	0.76±0.08	0.168±0.009	3.80±0.12	0.145±0.005
	234	16.2±0.4	0.93±0.06	0.169±0.007	4.30±0.16	0.149±0.013
	235	16.5±0.4	0.95±0.11	0.161±0.009	4.36±0.19	0.124±0.008
	Mean	16.3±0.3	0.88±0.05‡	0.166±0.005‡	4.15±0.09‡	0.139±0.005‡
Hypophysectomized plus Growth Hormone	241	16.1±0.3	1.89±0.10	0.257±0.008	6.06±0.26	0.182±0.007
	242	16.5±0.3	1.64±0.14	0.221±0.008	5.03±0.15	0.158±0.008
	244	15.1±0.2	1.72±0.14	0.250±0.010	4.65±0.21	0.151±0.007
	Mean	15.9±0.2	1.75±0.07‡	0.243±0.005‡	5.25±0.12	0.164±0.004

* Expressed in arbitrary units/nucleus—see Swift (1950). Each value represents a mean of 15 measurements.
† Expressed as extinction. Each value represents a mean of 15 measurements.
‡ These values are significantly different from control values ($P<0.01$).

cytochemical techniques are recorded in Table 3. A decrease in both nuclear and cytoplasmic RNA and protein was observed following hypophysectomy. It should be noted that whereas nuclear values represent content modification, the cytoplasmic values represent concentration changes. Since the liver cells of hypophysectomized rats were smaller than those of the controls, the changes in cytoplasmic concentration reflect a greater absolute change than is indicated in Table 3. Cytochemical analyses of both nuclei and cytoplasm show that growth hormone treatment of hypophysectomized rats results in an accumulation of RNA greater than observed in the control animals. The cellular protein however, is restored only to control levels.

The ultraviolet absorption measurements of cytoplasmic RNA were confirmed by Azure A staining following ribonuclease digestion. Staining of nuclei with methyl green established that no "depolymerization" of DNA occurred as a result of hypophysectomy (values expressed in arbitrary units found for control and hypophysectomized animals were 9.75 ± 0.11 and 9.74 ± 0.09 respectively).

In order to compare the results obtained by the various techniques employed it was desirable to express all data in terms of percentage deviations from control values (Table 4). Since the cytoplasmic figures in Table 3 are a measure of concentration, it was necessary to convert these extinction values into arbitrary units reflecting cytoplasmic content. This was accomplished by obtaining a reasonably accurate measure of cytoplasmic volume which was then used to convert the extinction values found experimentally to arbitrary units of content (Swift 1950).

TABLE 4. PERCENTAGE DEVIATIONS FROM INITIAL VALUES EXPRESSED AS 100 PER CENT

	Method employed					Volume	
	Chemical	Microspectrophotometric					
	RNA	RNA		Protein			
	Whole tissue	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Controls	100	100	100	100	100	100	100
Hypox	72.1	63.8	70.2	80.6	58.6	73.1	68.0
Hypox & GH	95.5	126.9	112	101.9	94.8	103.1	92.7

Table 4 reveals an interesting correlation between the cytochemical and chemical methods of analysis. The decrease in RNA is of the same order of magnitude in the hypophysectomized animals employing either technique. However, examination of the data for hypophysectomized animals which received growth hormone shows that by the cytochemical method there is a significant ($P<0.01$) increase in RNA above control levels. This is not

evident from chemical analysis. At present we can offer no satisfactory explanation, however, it should be pointed out that the cytochemical measurements are made on selected parenchymal liver cells, while chemical analyses are carried out on gross tissue samples which consist of a number of cell types.

Cytochemical observations of RNA indicate that the changes in the nuclei and cytoplasm are comparable. It is of interest to note that protein content in both nucleus and cytoplasm deviates in the same direction as RNA. It is apparent (Table 4) that the protein content of the cytoplasm is altered by hypophysectomy to a greater degree than is that of the nucleus. In hypophysectomized growth hormone-treated animals these protein levels are not significantly different from those of control animals.

From this table it may also be noted that, along with the decrease in RNA and protein fractions of the nucleus and cytoplasm resulting from hypophysectomy, there occurs a parallel change in nuclear and cytoplasmic volume. In liver cells of control and hypophysectomized animals injected with growth hormone, no volume differences were observed.

DISCUSSION

It should be pointed out that chemical analysis reveals the concentration of nucleic acids per unit weight of tissue. According to this method a reduction in cell size unaccompanied by an alteration in DNA per nucleus would result in an increase in DNA per unit weight of tissue. Davidson and Leslie (1950) have recently called attention to the relative constancy of DNA per nucleus and have suggested that, when chemical methods are employed, all data should be calculated on the basis of a constant DNA. However, in view of the evidence presented by Diermeier *et al.* (1951, 1952) Ely and Ross (1951) and Leuchtenberger and Schrader (1952) this recalculation may not be permissible in all instances. By cytochemical techniques, on the other hand, nucleic acid changes at the cellular level can be determined. Although ordinarily absolute amounts of nucleic acids are not obtained by this procedure, relative changes in content or in concentration are quantitatively determinable. In addition, this method permits analysis of the predominant functional cells of a tissue, readily excluding blood vessel cells, connective tissue cells, tissue macrophages and other such cells.

Capersson (1950), in his monograph, suggests that nuclear RNA is of particular importance in protein synthesis, while Brachet, in a recent review (1950), presents evidence suggesting that cytoplasmic RNA is more closely associated with this process. Clearly, little evidence on this point can be obtained by chemical analysis of whole tissue, since the result of such an analysis does not distinguish between cytoplasmic and nuclear

RNA. The microspectrophotometric methods, however, show unequivocally that hypophysectomy causes a fall in both nuclear and cytoplasmic RNA, and that growth hormone treatment of hypophysectomized rats results in supernormal levels of both nuclear and cytoplasmic RNA. On the basis of these data, then, the protein synthetic reactions instituted or implemented by growth hormone therapy cannot be said to be associated predominantly with one or the other intracellular RNA pool.

The concept that there is a relationship between protein synthesis and RNA concentrations is clearly supported by these data, as it is by the data of Geschwind *et al.* (1950). The depletion of nuclear and cytoplasmic protein and of nuclear and cytoplasmic RNA in the livers of hypophysectomized rats has been clearly demonstrated. The reversal of these defects by a purified pituitary growth hormone preparation has been described. No conclusions concerning the relationships of nuclear RNA and protein, and cytoplasmic RNA and protein are warranted by these data at this time. It is possible, however, that time curves of these responses to growth hormone injection in hypophysectomized rats not only will yield interesting information about the role of nucleic acids in protein synthesis but also will suggest a possibly fruitful hypothesis concerning a biochemical locus of action of pituitary growth hormone.

SUMMARY

Hypophysectomy results in (1) a marked fall in nuclear and cytoplasmic RNA of rat liver cells; (2) a marked decrease in nuclear and cytoplasmic protein content and (3) no change in DNA per nucleus of these cells. The liver cells of hypophysectomized rats treated with a purified growth hormone preparation show normal amounts of protein and increased amounts of both nuclear and cytoplasmic RNA as compared with controls.

Chemical analysis of the same material shows an apparent increase in DNA per unit weight of tissue. When the chemical data are recalculated on the basis of a constant DNA content, the RNA decrease after hypophysectomy is consistent with that observed cytochemically. These data are summarized in Table 4.

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THE EFFECT OF AMPHENONE-B ON THE CHOLESTEROL AND ASCORBIC ACID CONTENT OF THE RAT ADRENAL

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PREVIOUS publications have described the chemical synthesis (Allen and Corwin, 1950) and some of the biological properties of Amphenone-B.* This substance has progestational activity in the rabbit (Allen, Hertz and Tullner, 1950) and has an anesthetic effect in the rat, rabbit and dog (Hertz, Allen, Tullner and Westfall, 1952). Amphenone-B also has folliculoid activity (Hertz, Allen and Tullner, 1950) in the castrated female rat. It has been reported, in addition, that the rat responds to the compound with a marked hyperplasia of the thyroid, a goitrogenic action which is probably related to its aromatic amino groups (Hertz *et al.*, 1951). But of all the effects in its bizarre spectrum of endocrine action, the most interesting and unexpected is the profound hypertrophy of the adrenal glands. Just as the goitrogenic activity can be suppressed by the simultaneous administration of thyroxine, the hypertrophy of the adrenals can be suppressed by the administration of cortisone (Hertz, Tullner, and Allen, 1951). Recently, it was reported that the increase in the weight of the adrenal of the rat in response to Amphenone is associated with a marked increase in the adrenal cholesterol (Hertz, 1951; Hogness *et al.*, 1952). In the present report we wish to describe more fully the nature of the increase in adrenal cholesterol, and the response of the ascorbic acid and cholesterol content of such glands to stress.

MATERIALS AND METHODS

All of the animals used in these experiments were female Holtzman rats. Some of the animals had been spayed 30–90 days prior to the onset of Amphenone treatment in order to assess the estrogenic activity of the compound. The control and treated groups were of similar age and weight in each instance, and the weight and the time of previous ovariectomy are indicated in the footnotes appended to each table.

In the first four series of tests (Tables 1–4), where the effect of stress was not evaluated, the animals were killed with ether, the adrenal glands removed and weighed on a

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* 1,2 *bis*-(p-Aminophenyl)-2-methylpropanone-1 dihydrochloride.

Roller-Smith torsion balance. The adrenals were immediately placed in a McShan homogenizer containing one ml. of 25% ethyl alcohol, homogenized and sufficient 25% alcohol added to bring the final concentration to 10 mg. per ml.

In the tests for the effect of stress (Table 5) the right adrenal was removed through a flank incision under ether anesthesia and prepared as described above. Upon recovery from anesthesia, (a few minutes), the animal was placed in a cold room at 5° C. for four hours, then killed with ether. The left adrenal was removed immediately and similarly prepared for assay.

Cholesterol was determined by two procedures, both modifications of the method described by Sperry (1938). The first procedure included the turbidimetric estimation of the digitonide after precipitation under conditions essentially similar to his. For this

TABLE 1. EFFECT OF VARIOUS DOSAGES[1]

Dose (mg./100 gm. body wt./day)	Number animals[2] per group	Wet weight both adrenals (mg.)	Mean ascorbic acid conc. (mg./gm.)	Mean cholesterol conc. (mg./gm.)
Five days of treatment				
0	5	48 ± 6	4.25 ± .30	41 ± 3.5
5.0	5	53 ± 6	4.43 ± .24	50 ± 4.0
10.0	5	63 ± 3	4.52 ± .34	55 ± 5.0
20.0	4[3]	116 ± 5	3.63 ± .07	93 ± 12.5
Ten days of treatment[4]				
0	5	60 ± 7	3.96 ± .30	46 ± 5
2.5	5	61 ± 11	4.44 ± .80	59 ± 10
5.0	5	67 ± 8	4.59 ± .15	54 ± 10
10.0	5	89 ± 16	4.47 ± .35	85 ± 10
20.0	5	137 ± 31	3.00 ± .35	75 ± 16

[1] Amphenone B dihydrochloride administered once daily by stomach tube in 0.5 cc. water.
[2] Female Holtzman rats, spayed 25 days prior to onset of therapy, weighing 115–124 gms. at onset of treatment.
[3] A fifth animal died during treatment.
[4] Animals in this series weighed 176–288 grams at the onset of treatment, and had been spayed 55 days previously.

purpose one ml. of the above homogenate was treated with 9 ml. of 1:1 acetone-alcohol. Following this heat treatment, separation of protein, saponification of 2 ml. aliquot of the protein filtrate and acidification, 2 ml. of 0.2% digitonin in 1:1 acetone-alcohol and 2 ml. of water was added [the appearance of a precipitate before the addition of water was a convenient indication of insufficient acidification and could be corrected at this point with an additional drop of glacial acetic acid]. After mixing and standing for about 90 minutes the turbidity could be read at 400 millimicrons in a spectrophotometer. This simple procedure was effective for normal adrenals but with adrenals of high lipid content a non-specific extra turbidity was encountered which necessitated for its suppresion the addition of one ml. of ether (or acetone) 15 minutes after the addition of the water. Consequently, all samples were so treated.

This method gave results that were fairly comparable with those employing the Liebermann-Burchard color reaction, with the advantage of stability in the turbidimetric readings. However, occasional variability, the source of which could not be identified immediately, led to its temporary disuse pending further study. Frequent checks were made and all later results were obtained using aliquots of similar acetone-alcohol extracts

evaporated to dryness after saponification and treated according to Sperry (1938) omitting the digitonide separation.

Ascorbic acid was determined by the method of Roe and Kuether (Roe and Kuether, 1943) with previously described modifications (Schricker, Hertz and Tullner, 1951).

RESULTS AND DISCUSSION

The results are summarized in the accompanying tables. Each number represents the mean of five or more separate determinations.

Dosage. Table 1 indicates that a daily dosage of 20 mg./100 gm. body weight is necessary to produce a significant increase in the adrenal weight or cholesterol content in five days. If a daily dosage of 10 mg./100 gm.

TABLE 2. EFFECT OF DURATION OF TREATMENT WITH AMPHENONE-B[1]

Days of treatment	No. rats[2] per group	Mean control body wt.[3] (gms.)	Mean treated body wt. (gms.)	Mean control adrenal wt. (mg. wet wt. both glands)	Mean treated adrenal wt. (mg. wet wt. both glands)	Mean control ascorbic acid conc. (mg./gm.)	Mean treated ascorbic acid conc. (mg./gm.)	Mean control cholesterol conc. (mg./gm.)	Mean treated cholesterol conc. (mg./gm.)
5	5	55±4	35±5	18±1	26± 1	3.9±0.4	2.7±0.2	25±6	29± 6
10	5	71±4	54±1	22±3	41± 3	2.9±0.2	2.9±0.5	32±5	120± 7
22	5	82±4	63±2	23±3	52± 6	4.1±0.8	2.9±0.6	39±5	142±11
32	5	95±3	69±9	30±2	65±10	3.4±0.2	2.7±0.2	40±2	135± 5
36	5	105±4	73±7	29±3	65±12	5.3±0.4	3.3±0.6	44±9	155± 9
43	5	107±4	68±5	34±2	66± 6	4.2±0.4	2.7±0.5	66±9	172±16

[1] Given as 0.5% by weight of diet.
[2] Female Holtzman rats, 23 days old, weighing 37–42 gms. at onset of treatment.
[3] Pair-fed with treated animals. Diet: (daily average intake—6 gms.) 0.5% whole dried pork liver added to diet of Franklin *et al.*, J. Biol. Chem., *169*, 427, 1947.

body weight is continued for ten days, it produces an appreciable increase in both adrenal weight and cholesterol content, but less than that produced by 20 mg./100 gm. It should not be concluded that smaller doses are totally without effect, but that such minimal responses are highly variable.

Duration of Treatment. Table 2 illustrates the fact that, while the adrenal weight becomes somewhat reduced after 30 days of treatment, the increase in cholesterol concentration is progressive through the 43rd day and reaches as much as 17% of the wet weight of the adrenal.

Cholesterol Content of Other Tissues. Table 3 presents data concerning the effect of Amphenone B on the cholesterol content of tissues other than the

TABLE 3. EFFECT OF AMPHENONE-B[2] ON VARIOUS RAT TISSUES
(Cholesterol, mg./gm. ±Standard Deviation)

Treatment	Animals[1] per group	Serum	Liver	Kidney	Skeletal muscle	Brain	Adrenal
Control	5[3]	.67±.13	9.8±4.5	6.7±1.0	4.7±2.1	12.1±1.7	43.3±5.0
Amphenone 20 mg./100 gm.	5[4]	.60±.09	7.2±2.0	7.2±1.9	3.9±1.2	11.4±0.4	140.6±8.0

[1] 100 day old intact female Holtzman rats, averaging 178 gms. body wt.
[2] 20 mg./100 gm. body wt. by stomach tube.
[3] Average wt. at autopsy 205 gms.
[4] Average wt. at autopsy 173 gms.

adrenal. It will be seen that the effect upon adrenal cholesterol is unique and that the other tissues studied were not similarly affected.

Effect of Other Goitrogens and Estrogens. Table 4 illustrates experiments designed to test the possibility that the increase in adrenal cholesterol may be due to estrogenic activity in general or to hypothyroidism, or both. It is seen that large doses of estrone and thiouracil do not produce significant changes, and that the result of the simultaneous administration of Amphe-

TABLE 4. EFFECT OF THIOURACIL PLUS ESTRONE[1] COMPARED WITH AMPHENONE-B[2]

Treatment	Number of animals per group[3]	Wet wt. both adrenals (mg.)	Mean ascorbic acid conc. (mg./gm.)	Mean cholesterol conc. (mg./gm.)
Control	5	46 ± 4	4.1 ± 0.4	39 ± 5
Thiouracil-Estrone	5	32 ± 8	3.4 ± 0.3	31 ± 5
Amphenone-B	5	94 ± 4	2.9 ± 0.3	142 ± 12
EFFECT OF AMPHENONE-B[4] AND THYROXINE[5] GIVEN TOGETHER AND SEPARATELY				
Control	5[6]	64 ± 18	4.3 ± 0.2	45 ± 16
Thyroxine	5	65 ± 6	4.0 ± 0.4	46 ± 10
Amphenone-B	5	128 ± 51	3.6 ± 0.6	70 ± 17
Amph-B plus Thyroxine	5	160 ± 40	2.6 ± 0.5	72 ± 22

[1] Given as 2 mcgm. Estrone in 0.2 cc. corn oil subcutaneously daily for 17 days; thiouracil fed as 0.1% of diet.
[2] Fed as 0.5% diet. Controls pair-fed.
[3] 23 day old intact female Holtzman rats, weighing 40 gm. at onset of treatment.
[4] 20 mg./100 gm. body wt. daily for 10 days by stomach tube in 0.5 cc. H_2O.
[5] 20 mcgm./100 gm. body wt. daily for 10 days injected subcutaneously in 0.2 cc. 0.05 N NaOH.
[6] Female Holtzman rats, weighing 180–219 gms. at onset of treatment.

none and thyroxine differs in no way from the result of administration of Amphenone alone. Experiment C in Table 5 illustrates that estradiol in massive dosage does not produce changes similar to those seen following Amphenone.

Mobility of Adrenal Cholesterol. Reference to the data in Table 5 shows that the large accumulation of cholesterol in the Amphenone-treated gland does not decrease after the animals have been subjected to the acute stress of 4 hours exposure at 5° C. This degree of stress was sufficient to cause approximately a 50% depletion in both the concentration and total amount of adrenal cholesterol in the controls. There was no appreciable decrease of cholesterol in the adrenals of the amphenone-treated group. Treatment with estradiol caused no deviation in cholesterol response from that seen in untreated animals.

The ascorbic acid content of the adrenals of Amphenone-treated animals is consistently below the normal level. In addition, this ascorbic acid content is further reduced upon exposure to cold. This indicates that the

TABLE 5. EFFECT OF ACUTE STRESS[1] UPON ADRENAL ASCORBIC AND CHOLESTEROL CONTENT

Experiment	Treatment	No. rats[2] per group	Mean ascorbic conc.		Mean cholest. conc.		Mean adrenal weight		Total adrenal cholest.		Net change
			Prior to stress (mg./gm.)	After stress (mg./gm.)	Prior to stress (mg./gm.)	After stress (mg./gm.)	Prior to stress (mg./gm.)	After stress (mg./gm.)	Prior to stress (mg.)	After stress (mg.)	Total adrenal cholesterol (± mg.)
A	Control	5	2.4±0.2	1.7±0.2	43±12	21±12	31± 3	34± 1	1.36±0.5	0.72±0.4	−0.64±0.3
	Amphenone[4]	5	1.6±0.1	.9±0.3	147±14	152±22	69± 5	74± 4	10.11±1.5	11.26±2.2	+1.15±1.0
B	Control	5	1.7±0.4	1.1±0.0	34± 2	14± 5	28± 4	31± 2	0.97±0.2	0.43±0.17	−0.54±0.26
	Amphenone[4]	5	1.7±0.2	.6±0.0	114±31	123±30	65±15	66±13	7.29±2.6	7.66±2.1	+0.37±0.36
C	Control	5	4.7±0.2	2.4±0.2	42± 5	20± 5	32± 7	33± 8	1.33±0.24	0.63±0.11	−0.70±0.21
	Amphenone[4]	5	2.7±0.5	1.7±0.2	150±14	137±13	58±12	66±13	8.62±1.72	9.10±0.23	+0.48±2.7
	Estradiol[3]	5	3.9±0.2	2.2±0.1	37± 5	19± 2	24± 1	26± 1	0.90±0.11	0.50± .04	−0.40±0.3

[1] 4 hours at 5° C.
[2] Intact female Holtzman rats, averaging 180 gms.
[3] 2.0 micrograms in 0.2 cc. corn oil subcutaneously for10 days.
[4] 20 mg./100 gm. body wt. daily by stomach tube for 10 days.

cholesterol response and ascorbic acid depletion are readily dissociated in Amphenone-treated animals.

It would appear, then, that a direct or indirect action on adrenal cholesterol in the rat must be added to the previously reported estrogenic, progestational, goitrogenic and sedative properties of Amphenone-B. The action does not seem to be a resultant of the other properties, nor would it appear to be merely an effect on cholesterol metabolism in general.

In any given period of treatment the adrenal cholesterol is increased in proportion to the dose of Amphenone; and, while there is some irregularity in the first week, the adrenal cholesterol is proportional to the duration of treatment at any given dose.

The only other experimental condition in which the adrenal cholesterol of the rat has been raised to levels comparable with those observed following Amphenone is that described by Carroll (1951). He reported adrenal cholesterol values of 15 to 17% in rats fed rape seed oil. Carroll and Noble (1952) observed similar effects in rats fed erucic acid.

SUMMARY

Administration of Amphenone-B to rats causes an increase in both the concentration and total amount of cholesterol in the adrenal glands of the rat. In response to acute, severe stress produced by exposure to 5° C. for 4 hours, these animals show no depletion of adrenal cholesterol, although the expected depletion of ascorbic acid is seen.

The cholesterol content of serum and other tissues is unaffected by Amphenone. The elevation of adrenal cholesterol is not produced by estrone combined with thiouracil, indicating that Amphenone does not exert its adrenal effect by virtue of its folliculoid or goitrogenic properties.

The data indicate that Amphenone produces a marked derangement of sterol metabolism in the rat adrenal.

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THE HYPERTENSIVE EFFECT OF COMPOUND F ACETATE (17-OH-CORTICOSTERONE-21-ACETATE) IN THE RAT*

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A CONSIDERABLE volume of investigation in man as well as in animals has indicated that the adrenal cortex might be involved in hypertensive disease, either in a causal or supportive rôle (Soffer, 1948; Perera, 1950). Some impetus to these studies has been given by the exploration of the hypertensive properties of desoxycorticosterone acetate (DCA) (Selye, Hall and Rowley, 1943; Friedman, Polley and Friedman, 1948). In view of these findings, it seemed of importance to us to determine whether any compounds, either by reason of their structural or physiological properties, might oppose the known cardiovascular-renal effects of DCA. To this end we have set up a simple screening test and have thus far explored the action of progesterone, testosterone, estradiol, 21-acetoxy pregnenolone, "12-keto DCA," Compound S (11-desoxy-17-hydroxycorticosterone acetate), Compound A (11-dehydrocorticosterone), cortisone, hyaluronidase, Lipo-Adrenal Cortex (Upjohn) and pitressin. Of these, only Lipo-Adrenal Cortex, in large doses, and pitressin have thus far shown any ability to oppose these actions of DCA (Friedman and Friedman, 1950; Friedman and Friedman, 1951; Friedman, Friedman and Nakashima, 1952).

Recently, we have had available for testing Compound F acetate (17-OH-corticosterone-21-acetate) and our results form the substance of this report. As far as we can determine, no reports of the effects of continued administration of this steroid on the blood pressure have yet appeared.

EXPERIMENT 1

Methods. Thirty-two male albino rats weighing an average of 65 gm. were divided into 4 groups of 8 animals each. Group 1 served as untreated control while group 2 received DCA as pellets ($\frac{1}{4}$ of a 75 mg. Cortate pellet) administered as 2 on the first day of the experiment and one every fourth day thereafter to a total of 5 pellets in 2 weeks. Group 3 received 2 mg.

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of Compound F acetate daily by subcutaneous injection. This dose of Compound F, in microcrystalline form, was contained in 0.08 cc. of saline with added suspending agents and 1.5% benzyl alcohol as preservative. Group 4 received both DCA and Compound F, each in the amount and form as given to the two preceding groups.

At the conclusion of 3 complete weeks of treatment, blood pressure was determined by direct electromanometry using the Sanborn electromanometer with direct cannulation of the aorta with a 20 gauge needle under light ether anesthesia. Results obtained with this method are discussed elsewhere (Friedman, Friedman and Nakashima, 1952). Immediately upon

TABLE 1

Group	Control	DCA	Compound F	DCA + Compound F
Initial wt., gms.	61 ± 11	67 ± 8	66 ± 7	66 ± 10
Final wt., gms.	174 ± 22	175 ± 15	73 ± 8 ($p < 0.01$)	81 ± 23 ($p < 0.01$)
Blood Pressure, mm. Hg				
Systolic	118 ± 14	120 ± 17	147 ± 22 ($p < 0.01$)	152 ± 23 ($p < 0.01$)
Diastolic	66 ± 8	59 ± 15	79 ± 23	89 ± 22 ($p < 0.02$)
Mean	86 ± 11	81 ± 19	109 ± 23 ($p < 0.02$)	115 ± 26 ($p < 0.01$)
Pulse	53 ± 10	61 ± 9	68 ± 12 ($p < 0.02$)	64 ± 15
Heart wt., mg./100 cm.²	237 ± 21	253 ± 16	301 ± 14 ($p < 0.01$)	303 ± 13 ($p < 0.01$)
Kidney wt., mg./100 cm.²	457 ± 47	563 ± 26 ($p < 0.01$)	605 ± 27 ($p < 0.01$)	660 ± 95 ($p < 0.01$)
		↑——$p < 0.01$——↑		
Adrenal wt., mg./100 cm.²	7.49 ± 0.46	7.01 ± 0.69	3.86 ± 0.33 ($p < 0.01$)	3.74 ± 0.34 ($p < 0.01$)

In this and table 2 average and standard deviation are given as basic data. Probability calculation, unless otherwise indicated, refers to a comparison with the control mean.

conclusion of the pressure recording the animals were killed and hearts· kidneys and adrenals fixed for further study.

Observations. The results obtained are presented in table 1. It is apparent that Compound F caused a marked suppression of body growth, an effect in no way opposed by DCA. Despite this effect, and the obvious debility of the animals, Compound F caused a marked elevation in systolic and mean blood pressure with some elevation in diastolic pressure as well. Pulse pressure was, as a result, also significantly increased. DCA given alone had not yet affected blood pressure levels in this experiment although there is a suggestion that, if anything, when given with Compound F it tended to aggravate the hypertensive action of the latter. Sample records obtained are shown in figure 1.

These effects on the blood pressure were substantiated by the heart weights, for while DCA given alone had caused at this stage only the sug-

gestion of an increase, a marked increase in cardiac mass was observed in the groups receiving Compound F.

The increase in renal mass ordinarily caused by DCA was observed again in this experiment, with a more pronounced effect in the same direction being produced by Compound F. Similarly, the reduction in adrenal size ordinarily caused by DCA was almost negligible in comparison with the marked effect produced by Compound F.

It should be emphasized that organ weight studies may be misleading in view of the marked inhibition of general body growth caused by Compound F.

Compound F caused no distinct histological alterations in the kidney.

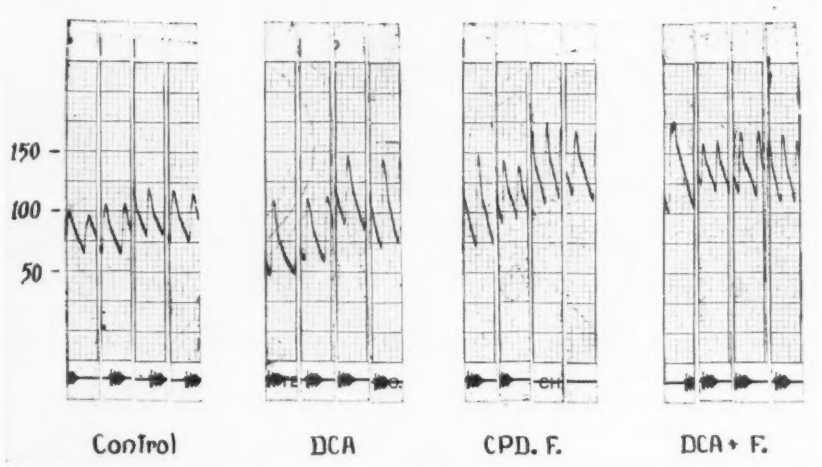

FIG. 1. Representative electromanometric tracings from 4 animals in each group of Experiment 1.

EXPERIMENT 2

Methods. The foregoing experiment had demonstrated that Compound F in the dosage and for the duration of the procedure used is markedly hypertensive in the rat. It appeared of importance, therefore, to make a direct assessment of the potency of this agent as compared with DCA. For this, both agents were given in the same dosage and medium in the experiment now to be described. Further, an attempt was made to obviate the complication of the effects of body growth by using larger animals and a smaller dose of Compound F.

Twenty-four male albino rats weighing an average of 146 gms. were divided into 3 groups of 8 animals each. Group 1 served as untreated control, the animals of group 2 received a daily subcutaneous injection of DCA

and those of group 3 received Compound F acetate similarly. Both steroids were administered as microcrystals in saline with added suspending agents and 1.5% benzyl alcohol as preservative. In each case, 1 mg. was injected daily.

At the conclusion of 2 complete weeks of treatment, a duration selected in order to compare the minimal effects of the 2 steroids under test, blood pressure was determined by direct electromanometry. At the conclusion of this test, arterial blood was taken for the determination of plasma

TABLE 2

Group	Control	DCA	Compound F
Initial wt., gms.	141 ± 11	144 ± 15	151 ± 9
Final wt., gms.	197 ± 15	184 ± 38	178 ± 16
Blood Pressure, mm. Hg			
Systolic	119 ± 14	131 ± 14	140 ± 13 ($p < 0.01$)
Diastolic	61 ± 13	74 ± 18	84 ± 17 ($p < 0.01$)
Mean	83 ± 15	93 ± 19	100 ± 19
Pulse	58 ± 6	58 ± 9	55 ± 7
Heart wt., mg./100 cm.^{2}	255 ± 20	284 ± 16 ($p < 0.01$)	277 ± 20 ($p < 0.05$)
Kidney wt., mg./100 cm.^{2}	449 ± 38	487 ± 26 ($p < 0.05$)	467 ± 34
Adrenal wt., mg./100 cm.^{2}	8.34 ± 0.53	7.38 ± 1.92	4.55 ± 0.89 ($p < 0.01$)
Plasma K mEq/L.	5.42 ± 0.98	5.09 ± 0.47	6.74 ± 1.38
Plasma Na mEq/L	147.7 ± 4.7	145.3 ± 2.2	143.8 ± 6.1

sodium and potassium, and the animals were killed. Hearts, kidneys and adrenals were fixed for further study.

Observations. The results obtained are presented in table 2. It is evident that Compound F in the dosage used in this experiment caused only a minor inhibition of body growth. While DCA caused only a non-significant tendency towards hypertensive blood pressure levels, Compound F in the same dosage and after only 2 weeks of treatment had already caused a significant elevation in both systolic and diastolic blood pressures. The tendency towards an increase in cardiac mass is evident in both groups, as is the tendency towards an increase in kidney mass. These increases are more evident in the group receiving DCA. The marked reduction in adrenal size caused by Compound F in the previous experiment is still evident with this smaller dose.

The effect on plasma sodium and potassium is equivocal at this time.

DISCUSSION

It is evident that Compound F acetate in adequate dosage causes a rise in blood pressure in the rat. It seems, too, that this steroid is at least as active and, indeed, probably more active in this respect than is DCA. While many features of the mechanism of action of Compound F remain

to be explored, the findings reported here have implications in themselves in the study of hormonal factors in hypertension. To date, most of the work of exploring the possible rôle of the adrenal cortex has made use of, or at least leaned heavily on, the results obtained with DCA. It has always been pointed out, however, that DCA is synthetic and probably not a normal secretion of the adrenal cortex to any appreciable extent if at all. Accordingly, Selye (1950) thought it important that Compound S in his hands had hypertensive effects. These effects were, however, apparently of a minimal nature, for they did not show up in our screening test (Friedman and Friedman, 1951) and when compared directly with DCA by Hall, Hall and McCleshey (1952) Compound S appeared to be much less potent that DCA.

With regard to the general question of adrenal participation in hypertension, our attention has recently been called to the fact that the hypertension which Grollman and his colleagues have been able to elicit in the bilaterally nephrectomized dog does not depend on the presence of the adrenal. Turner and Grollman (1951) have generalized from this that "the adrenals, contrary to widely current opinion, do not mediate the development of hypertension." It seems to us, however, that such a statement is unjustified, for it has not been proved that all forms of hypertension are similar in etiology—indeed, it is generally conceded that they are probably not. A large body of data, both clinical and experimental, supports the view that the adrenal participates in some forms of hypertension and it is with relation to this that Compound F requires further study.

SUMMARY

Compound F acetate has been tested for its cardiovascular-renal effects in the rat. This steroid appears to be a more potent hypertensive agent than DCA.

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THE DISTRIBUTION OF THYROXINE IN RAT LIVER CELL FRACTIONS[1]

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INTRODUCTION

EARLY information related to the distribution of iodine, iodine-containing substances, and thyroxine derivatives in the tissues and organs of a variety of animals has been summarized by Salter (1940). More recently Gross and Leblond (1947) have studied the distribution of administered I^{131}-labeled thyroxine in the tissues and organs of the rat. The localization of the iodine or thyroxine within the cell has not been investigated. The application of the method of Hogeboom, Schneider and Pallade (1948) and Schneider and Hogeboom (1950) for the differential fractionation of cellular constituents, in conjunction with the use of labeled thyroxine, has made possible a study of the distribution of thyroxine in the rat liver cell.

Although the thyroxine concentration was found to vary in different fractions of the liver cell, the ratio of thyroxine concentration to dry weight was approximately the same for each of the fractions studied.

METHODS

Two types of labeled thyroxine were employed in the present study: (1) thyroxine labeled with I^{131}, and (2) thyroxine labeled in the carboxyl position with C^{14}. These compounds were prepared according to the methods of Wang, Hummel and Winnick (1952).[4] The I^{131}-thyroxine was prepared from 3,5-diiodothyronine and hence (unless exchange occured), was labeled exclusively in the 3′,5′ positions. The specific activity of C^{14}-thyroxine was 5.3 μc./mg. and that of the I^{131}-thyroxine (about) 20 μc./mg

In order to express the radioactivity measurements in absolute units, the observed counts per minute were generally converted into corresponding μgm. of thyroxine by referring these measurements to standards containing known quantities of I^{131}- and

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[4] The I^{131} and C^{14} were obtained on allocation from the U. S. Atomic Energy Commission.

C^{14}-thyroxine.[5] The radiocarbon samples were corrected for self absorption by reference to a set of standards of varying layer thickness. Samples were generally counted (using a thin mica-window Geiger-Muller counter) to within a probable error of 2 to 3%, except for certain of the weaker C^{14}-samples in which the counting error was of the order of 5 to 8%.

Adult male Sprague-Dawley rats, weighing 300 to 350 grams, were used. Animals thyroidectomized at least 4 weeks previously were given single subcutaneous injections of 6.12 mg. of labeled thyroxine (4 mg. iodine) per kilo body weight at specified times prior to sacrifice. This level was based on the metabolic studies of Barker and Klitgaard (1952). The animals were maintained on a 5 hour feeding regime (Barker, 1949), and were usually fully post-absorptive at the time of sacrifice, 14 hours after the food was removed.

The rats were exsanguinated by removing 8 to 10 cc. of blood from the abdominal aorta into an oxalated syringe. The liver was perfused with 100 cc. of cold Krebs-Ringer solution through the portal vein. A weighed portion of the liver was minced, and homogenized (Potter and Elvehjem, 1936) in 3 volumes of 0.25 M sucrose. The homogenate was centrifuged at 670× gravity for 5 minutes, and the residue was again homogenized with 3 volumes of sucrose solution. The supernatant and rehomogenized sediment were recombined and diluted with the sucrose solution to make a 10% homogenate, based on the initial weight of liver. The homogenate was then fractionated by the method of Schneider and Hogeboom (1950). The entire procedure of mincing, homogenization and fractionation, was carried out at 2° to 4° C.

The fractions were precipitated with zinc hydroxide reagent (Somogyi, 1930), and washed three times with water. The precipitates were dehydrated with alcohol, 3:1 alcohol-ether, and ether, and then plated out on aluminum disks as described by Winnick (1950). Dry weight determinations on whole homogenates and liver fractions were performed using trichloroacetic acid as the precipitant, and then treating the protein precipitates with organic solvents as described above.

RESULTS

The distribution of I^{131}-thyroxine in the rat liver homogenate and in the cellular fractions is illustrated in Table 1. The thyroxine content of the homogenates declined continuously with time. C^{14}-Carboxyl thyroxine showed the same distribution at the 3 hour interval as was found for I^{131}-thyroxine.

The thyroxine contents of the various fractions comprise relatively fixed percentages of the total contained in the homogenates at the various time intervals studied. This is brought out in Table 1, in which the results are also expressed on a % basis. It is seen that the nuclear fraction contained approximately 15 to 22%, the mitochondrial or large granule fraction 19 to 28%, and the supernatant 44 to 60% of the thyroxine in the whole homogenates.

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[5] Although it is not certain that the radioactivities represent thyroxine *per se*, the very similar distribution of the two isotopes found in liver cells, together with much evidence accumulated by other investigators, makes it probable that the radioactivity is retained in the thyroxine molecule.

Table 2 gives the dry weight values of the cell fractions, expressed on a % basis, relative to the whole homogenate. The average values, 18.5, 27.4, and 52.1 for nuclear, large granule, and supernatant fractions, respectively, show a close similarity to the % distribution values of thyroxine shown in Table 1.

In a few experiments, the further fractionation of the supernatant into

TABLE 1. DISTRIBUTION OF I^{131}-THYROXINE IN RAT LIVER CELL FRACTIONS

Time after administration	Thyroxine content						
	Homogenate	Nuclear fraction		Large granule fraction		Supernatant fraction	
hrs.	μg./100 gm.	μg./100 gm.	%[1]	μg./100 gm.	%[1]	μg./100 gm.	%[1]
3	1342	265	18.4	287	23.9	757	53.8
	1220[2]	248[2]		284[2]		640[2]	
6	1164	261	22.4	328	28.2	509	44.0
12	945	201	15.5	163	19.2	579	57.9
24	524	84	17.3	97	20.7	345	61.7
Average			18.4		23.0		54.2

[1] In terms of the corresponding homogenate taken as 100.
[2] Value obtained with C^{14} thyroxine.

TABLE 2. FAT-FREE DRY WEIGHT OF LIVER CELL FRACTIONS

	Homogenate	Nuclear	Mitochondria	Supernatant	Total of fractions
Dry weight (mg.)[1]	178.2	33.0	48.8	92.9	173.2
Average % of homogenate	100	18.5	27.4	52.1	97.2

[1] Average of 6 experiments.

TABLE 3. DISTRIBUTION OF I^{131}-THYROXINE FOLLOWING *in vitro* ADDITION TO NORMAL RAT LIVER HOMOGENATE

Fraction	μgm. thyroxine/100 gm. wet weight liver	% of added thyroxine
Homogenate	2244	(100)
Nuclear	197	8.8
Mitochondria	260	21.8
Small Granule	579	25.8
Clear Supernatant	564	25.1

a small granule (microsome) fraction and an almost granule-free phase indicated that approximately 50% of the thyroxine present in the supernatant was associated with the small granules. The small granules were found also to contribute approximately 50% of the dry weight of the supernatant fraction.

The distribution of thyroxine in the cellular fractions following the

in vitro addition of thyroxine to a normal rat liver homogenate is indicated in Table 3. The distribution is essentially similar to that encountered after the *in vivo* administration of thyroxine. In this experiment the supernatant phase was again further fractionated to give a small granule fraction and clear supernatant. These two fractions together account for 51% of the thyroxine content of the homogenate, as compared to approximately 54% in the *in vivo* studies (Table 1, supernatant fraction).

An experiment designed to determine whether the thyroxine of one cellular fraction can redistribute itself among the other cell constituents is described in Table 4. Mitochondria containing labeled thyroxine, and

TABLE 4. ADDITION OF LABELED MITOCHONDRIA TO LIVER HOMOGENATE

2 ml. of the large granule fraction (representing 0.4 gm. of liver) from a rat previously injected with I^{131}-thyroxine, were mixed with 10 ml. of a freshly-prepared non-labeled 1:10 liver homogenate. The latter was immediately subjected to the usual fractionation procedure at 2–4°.

Fraction tested	μgm. of thyroxine	Thyroxine content
Initial labeled mitochondria	0.942	100
Labeled mitochondria plus unlabeled homogenate	0.962	102
Mixed mitochondria	0.175	18.5
Nuclear fraction	0.118	12.5
Supernatant fraction	0.572	60.6
Sum of 3 cellular fractions	0.865	91.6

washed with sucrose solution, were mixed with a normal rat liver homogenate and fractionated. It is seen that only 18.5% of the initial I^{131} remained in the large granule fraction, and that the greatest radioactivity was found in the supernatant phase. The observed redistribution is too extensive to be explicable on the basis of inadequate separations in the centrifugation procedure.

It may be pointed out that direct extractions of the fresh liver fractions with butanol yielded considerably lower radioactivities, as compared with the zinc proteinate method. The latter proved the most satisfactory of the techniques tested.

DISCUSSION

In studying the distribution of thyroxine in the cell, it has been assumed that the hormone penetrates the cell boundary. The following facts support the view that the intracellular distribution is not an artifact produced by the disruption of the cells, with a consequent redistribution of the thyroxine from the cell boundary into the cell: Taurog, Briggs, and Chaikoff (1952) have indicated that thyroxine appears in the bile in two forms, one being the unaltered thyroxine molecule, and the other a conjugated

form, which may be a β-glucuronide. Furthermore, $C^{14}O_2$ can be isolated from the expired air of rats administered C^{14}-carboxyl labeled thyroxine (Lipner, Klitgaard, Barker, and Winnick, 1952). Since detoxification and decarboxylation are typical intracellular phenomena, it is logical to assume that thyroxine penetrates into the liver cell.

The observed distribution of thyroxine in rat liver is most readily correlated with the organic material (probably protein) of the fractions, and not with surface areas, since in the latter situation there would be a relatively small quantity of thyroxine in the nuclear and large granule fractions, and a preponderance of thyroxine (in considerable excess of that found) in the supernatant fraction. The time intervals chosen in the present study cover the range of the early metabolic response of liver slices, following subcutaneous injection of thyroxine into rats. However, the data that have been presented do not offer any indication of where thyroxine may exert its effect in the liver cell, nor do they exclude the cell membrane as a possible site of action.

Winzler and Notrica (1952) have presented evidence for an association of thyroxine with one, or perhaps two specific proteins of blood plasma. In the case of liver, there are thus far no similar indications, and only a minor part of the thyroxine is attached to particle-free soluble proteins. The combination of thyroxine with proteins is a loose one, and is partially disrupted by butanol.

The distribution observed with the present zinc proteinate precipitation method is not dependent upon the intact cell, since a similar distribution was observed following the addition of thryoxine to a liver homogenate *in vitro*. Additional evidence is the observation that thyroxine can apparently shift readily from one cellular component to another.

SUMMARY

I^{131}-labeled thyroxine administered to thyroidectomized rats 3, 6, 12, or 24 hours prior to sacrifice, assumes a characteristic distribution in the rat liver cell. The nuclear, mitochondrial, and supernatant fractions accounted for 18, 23, and 54%, respectively, of the total thyroxine in the homogenates. Approximately half the thyroxine in the supernatant fraction was associated with the microsomes. Somewhat similar distributions were observed when either I^{131}-thyroxine or a labeled mitochondrial preparation (from a rat given I^{131}-thyroxine) were mixed with a normal liver homogenate *in vitro*.

There was no indication that thyroxine concentrates in any of the cellular fractions.

The available evidence indicates that thyroxine may, and does, enter the rat liver cell.

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EFFECTS OF TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES OF THE RAT

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MANN and Lutwak-Mann (1951) have reviewed the literature dealing with the many functional as well as structural changes which are known to occur in the male accessory organs of reproduction in mammals when the source of the testicular androgen, testosterone, is removed. The manner in which this hormone functions or the exact site of the biochemical lesion induced by its absence are unknown.

This investigation consists of a biochemical and physiological study of the normal development of one of the accessory organs, the seminal vesicles, of the rat as well as the effects of castration and hormone replacement on this organ of the castrate.

METHODS

Male rats of the Holtzman-Rolfsmeyer strain were used throughout this investigation. The animals were maintained in groups of four to eight in individual wire cages and were fed Purina Laboratory Chow with supplements of raw carrots and pork liver.

Animals were bilaterally castrated and the testes and epididymides were completely removed. An interval of at least twenty days was allowed to elapse after castration before hormone treatment. In all cases where testosterone propionate (Peranderen) was administered, it was given subcutaneously in daily doses of 500 μg.

The dry matter of secretion-free glands as well as the secretion was determined by heating samples of these materials at 105° C. overnight in an electric oven. Total nitrogen analyses were made by a standard micro-Kjeldahl procedure. The method of Folin and Malmros (1929) for blood sugar was used for the determination of total reducing substances present in seminal-vesicle secretion. Dry, secretion-free glands were extracted with ethyl alcohol followed by ethyl ether in a Soxhlet extractor and the lipid was determined gravimetrically following the removal of the solvent on a steam bath. Lipid analyses were made on the fresh secretion by the method described by Wilson and Hanner (1934). Ash analyses were carried out in a muffle furnace by heating the samples at 600° C. for four hours.

The method used in estimating acid and alkaline phosphatase activities was that described by Greenberg, Lucia, and Weitzman (1939) and as modified by Porter and Melampy (1952). A phosphatase unit as used here is equal to the mg. of tyrosine equivalent

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to the phenol liberated from disodium phenylphosphate per gm. dry weight of tissue per hour. In some of the data presented phosphatase activity is expressed as units per gland. For the measurement of catheptic activity of seminal-vesicle tissue the method of Anson (1936) was employed. The substrate was denatured bovine hemoglobin. After digestion the protein was precipitated by adding trichloroacetic acid and the tyrosine and tryptophan produced were determined colorimetrically, using the phenol reagent of Folin and Ciocalteu (1927). A catheptic unit is defined as the mg. of tyrosine equivalent to the tyrosine and tryptophan liberated per gland per hour. The Q_{O_2} was determined by the direct method, using the Warburg constant volume respirometer. In these determinations Krebs-Ringer-phosphate was used as the tissue medium and air was the gas phase.

RESULTS

The data obtained from various aspects of this investigation are presented in Tables 1–5. The change in weight and chemical composition of

TABLE 1. CHANGE IN WEIGHT AND COMPOSITION OF SECRETION-FREE SEMINAL VESICLES OF THE RAT DURING GROWTH

Group number	No. of animals	Body wt. gm.	Secretion wet wt. per gland mg.	Secretion-free seminal vesicles			
				Wet wt. mg.	Dry wt. mg.	Total nitrogen mg. (mean)	Total lipid mg. (mean)
1	10	77* ±1.3			2.1	0.3	0.1
2	10	94 ±1.8			2.2	0.3	0.1
3	8	146 ±1.4	5.5* ± 1.5	39* ± 3.5	8.9* ±0.9	1.2	0.3
4	7	188 ±1.9	77.0 ±18.1	99 ±11.7	23.0 ±2.7	3.2	1.2
5	7	258 ±1.6	361.0 ± 2.9	202 ±13.5	48.0 ±3.2	6.9	2.4
6	5	298 ±2.8	451.0 ±44.8	265 ±12.1	60.0 ±3.5	8.6	3.0
7	5	358 ±0.6	905.0 ±87.8	297 ±12.1	72.0 ±3.9	10.2	3.5
8	5	385 ±2.0	1201.0 ±131.3	390 ±24.7	96.0 ±6.7	13.6	4.4

* Mean and standard error.

the secretion-free seminal vesicles of the normal rat from puberty through sexual maturity are summarized in Table 1.

The effects of castration on weight, nitrogen content, and Q_{O_2} of secretion-free seminal vesicles of the rat and on the amount of secretion present in these glands at varying time intervals after castration are presented in Table 2. The effects of testosterone propionate on these same factors of the castrate rat are given in Table 3. The influence of castration on catheptic, acid phosphatase, and alkaline phosphatase activities of secretion-free seminal vesicles of castrate rats are shown in Table 4, and these same enzymatic activities as modified by testosterone propionate are presented in Table 5.

A group of 10 rats weighing 280 ± 4 gm. was used to obtain data on the composition of seminal vesicle secretion. There was 766 ± 54 mg. of secretion in the seminal vesicles of each animal. The dry matter of this secretion was 25.9 ± 0.3 per cent and the total nitrogen and reducing sub-

TABLE 2. EFFECT OF CASTRATION ON WEIGHT, NITROGEN CONTENT, AND Q_{O_2} OF SECRETION-FREE SEMINAL VESICLES OF RATS AND ON THE AMOUNT OF SECRETION PRESENT

Days after castration	No. of rats	Wet weight of secretion-free seminal vesicles mg.	No. of rats	Nitrogen content per gland mg. (mean)	No. of rats	Q_{O_2}† (mean)	No. of rats	Weight of secretion per gland mg.
0	10	284* ±14	7	6.9	2	2.7	10	766* ±54
2	10	185 ± 6	4	4.2	4	2.4	4	167 ±28
5	10	145 ±12	4	3.2	4	2.5	4	18 ± 2
10	10	109 ± 5	4	2.4	4	2.4	4	11 ± 1
20	4	77 ± 2	4	1.8	4	2.8	4	0
Wt. at castration (gm.)	250–300		250–260		250–260		250–300	

* Mean and standard error.
† c.mm. of oxygen per mg. dry weight of tissue per hour.

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON WEIGHT, NITROGEN CONTENT, AND Q_{O_2} OF SEMINAL-VESICLE TISSUE OF CASTRATE RATS AND QUANTITY OF SECRETION CONTAINED IN THE GLAND

Days of injection 500 μg. per day	No. of rats	Wet weight of secretion-free seminal vesicles mg.	No. of rats	Nitrogen content per gland mg. (mean)	No. of rats	Q_{O_2}† (mean)	No. of rats	Weight of secretion per gland mg.
0	5	48* ± 3	6	0.2	6	3.5	5	0
2	5	67 ± 4	6	0.4	6	6.2	5	22* ± 3
5	5	203 ± 9	5	1.5	5	4.2	5	107 ± 5
10	5	355 ± 9	5	3.4	5	3.8	5	677 ± 54
20	5	525 ±27	5	7.3	5	3.4	5	1322 ±113
Wt. at castration (gm.)	280–320		55–65		55–65		280–320	

* Mean and standard error.
† c.mm. of oxygen per mg. dry weight per hour.

TABLE 4. EFFECT OF CASTRATION ON CATHEPTIC, ACID PHOSPHATASE, AND ALKALINE PHOSPHATASE ACTIVITIES OF SECRETION-FREE SEMINAL VESICLES OF CASTRATE RATS

Days after castration	No. of rats	Catheptic units per gland	No. of rats	Acid phosphatase units per gland	No. of rats	Alkaline phosphatase units per gland
0	6	2.8* ±0.3	21	5.2* ±0.3	8	14.8* ±0.8
2	6	3.2 ±0.2	6	4.7 ±0.2	6	13.5 ±0.8
3	6	3.3 ±0.2				
5	6	2.2 ±0.1	5	3.1 ±0.4	6	9.1 ±1.0
10	6	1.9 ±0.2	6	3.3 ±0.2	6	6.0 ±0.5
20			6	1.8 ±0.2	6	2.8 ±0.3
24	6	0.9 ±0.1				
Wt. at castration (gm.)	245–270		245–260		245–260	

* Mean and standard error.

stances, calculated as glucose, were 13.6±0.1 and 0.35±0.01 per cent, respectively. The N.P.N. was 0.44 per cent on a dry-weight basis and the ash content was 1.6 per cent. There was no lipid in this secretion.

DISCUSSION

Male rats were selected at different stages of maturity to furnish data relative to the normal development of the seminal vesicles, particularly as related to the secretory activity. These data, presented in Table 1, indicated that while the average body weight of the animals in Group 5 was approximately 3 times greater than that of Group 1, the dry weight of the secretion-free seminal vesicles was 24 times greater. Furthermore, the

TABLE 5. EFFECT OF TESTOSTERONE PROPIONATE ON CATHEPTIC, ACID PHOSPHATASE, AND ALKALINE PHOSPHATASE ACTIVITIES OF SECRETION-FREE SEMINAL VESICLES OF CASTRATE RATS

Days of injection 500 μg. per day	No. of rats	Catheptic units per gland	No. of rats	Acid phosphatase units per gland	No. of rats	Alkaline phosphatase units per gland
0	6	0.9*±0.1	5	0.1*±0.1	5	1.3*±0.4
2	6	1.1 ±0.1	5	0.5 ±0.1	5	3.3 ±0.2
3	6	2.3 ±0.1				
5	6	3.3 ±0.2	5	3.1 ±0.1	5	38.3 ±3.0
10	6	4.5 ±0.2	5	9.5 ±0.7	5	50.4 ±2.5
20			5	11.5 ±0.4	5	62.8 ±2.6
Wt. at castration (gm.)	245–270		280–320		280–320	

* Mean and standard error.

body weight of the animals in Group 8 was only 5 times that of Group 1, the gland dry weight of the former was approximately 46 times that of the latter. The dry weight of the secretion-free seminal vesicles was a linear function of the body weight in animals weighing approximately 150 gm. or more. This relationship is expressed by the equation $Y = 0.3371x - 40.43$ and is significant at $P = 0.01$. The lack of development of the seminal vesicles of animals weighing less than 150 gm. was probably related to the low level of sex-hormone production in these animals. The percentage dry weight of the seminal-vesicle tissue indicated little, if any, change during development. There was a negligible amount of secretion in the glands of Groups 1 and 2; however, in comparing the amount of secretion present in the glands of Group 3 with those of Group 8, there was 218 times more secretion in the latter than the former. The total nitrogen content of the secretion-free glands of Group 8 was 45 times greater than Group 1. Also, the total lipid content of these organs showed a forty-four-fold increase.

As shown in Table 2, the weight of the seminal vesicles decreased 35

per cent 2 days after castration, 49 per cent after 5 days, 62 per cent after 10 days, and 73 per cent after 20 days. The decrease in the nitrogen content of the gland after castration paralleled the decrease in organ weight. The total nitrogen decreased 74 per cent by the twentieth day after castration. It is rather significant that the change from a secretory to a non-secretory state of seminal-vesicle tissue as affected by castration did not alter the Q_{O_2} of the secretion-free tissue. In the case of normal animals weighing 250 to 260 gm. at the time of castration, the Q_{O_2} was 2.7 and 20 days following castration the Q_{O_2} was 2.8. It appears from these data that the oxygen requirement, expressed as Q_{O_2}, of this tissue is not affected by the withdrawal of the male hormone. The importance of testosterone to the secretory activity of the seminal vesicles is indicated by the rapid fall in the amount of secretion in the gland following castration (Table 2). Two days after castration there was a 78 per cent decrease in the quantity of secretion initially present. Furthermore, the secretion had decreased to 98 per cent by the fifth day, 99 per cent by the tenth, and was completely absent by the twentieth day.

The influence of daily injection of 500 μg. of testosterone propionate on the seminal vesicles of castrate rats is shown in Table 3. There was a fourfold increase in the weight of the glands after 5 days of injection compared to the weights of the controls and after 20 days had increased 11 times. Likewise, there was a fivefold and sixtyfold increase in the amount of secretion present after 5 and 20 days of injection when compared to those receiving the hormone for 2 days.

The injection of testosterone propionate into castrate rats caused a significant increase in the Q_{O_2} of seminal-vesicle tissue two days after the beginning of the injection (Table 3). However, this significant rise (Sig. at $P = 0.01$) was followed by a decrease to the initial level by the end of 20 days of hormone treatment. There was no significant difference between the intial and final Q_{O_2} in these experiments. An explanation for this rise and its subsequent decrease is not apparent from the information at hand. However, the data of Rudolph and Samuels (1949) on the early effects of testosterone also showed a significant increase in the Q_{O_2} of seminal-vesicle tissue at 10 and 20 hours after hormone injection, but their observations did not extend beyond this time. The animals in their experiments received 1 mg. of hormone in a single dose. The animals used in the study of the effects of male sex hormone on the seminal vesicles were castrated when they weighed 55 to 65 gm. and they received their initial injection of testosterone propionate when they weighed 240 to 265 gm. The nitrogen content of these glands showed approximately a 37-fold increase during the period of hormone injection.

As shown in Table 4, castration induced little change in the catheptic

activity of the seminal vesicles during the first 3 days after castration. In evaluation of this fact it is important to stress that the total nitrogen content decreased approximately 40 per cent during the same period of time as calculated from data presented in Table 2. This indicates that the decrease in total nitrogen of the gland was not accompanied by a uniform reduction in all of the tissue protein, *e.g.*, enzymes. As a result it was possible for the enzyme(s) responsible for the catheptic activity to remain constant even under conditions of a decreasing protein content of the gland. However, there was a gradual decline in this activity during the period from 3 to 24 days.

In evaluating the changes occurring in acid phosphatase activity on a gland basis, there was a 40 per cent decrease 5 days following castration and by 20 days a 65 per cent reduction as shown in Table 4. Likewise, the alkaline phosphatase activity decreased 81 per cent. There was striking similarity between the decrease in the activities of these enzymes and the loss in weight of the glands as indicated in Table 2.

The importance of the male hormone in stimulating catheptic activity is indicated by the data in Table 5 which show a fivefold increase in catheptic activity of the seminal vesicles of castrates which had been injected for ten days. Likewise, this increase in catheptic activity of this gland was accompanied by a corresponding increase in total nitrogen as shown in Table 3. The nitrogen content of the gland increased 17 times in the castrate during the first 10 days of hormone injection. Rothschild and Junqueira (1951) have shown a significant relationship between male-sex hormone and catheptic activity of submaxillary glands of male mice. The acid phosphatase showed an increase during the first 10 days of injection and little change thereafter as indicated by the data in Table 5. The total alkaline phosphatase activity per gland of the castrate rat receiving testosterone propionate increased rather slowly during the first 2 days of injection but showed a marked rise between this time and 5 days. The activity of this enzyme at the end of the experimental period was 47 times greater than that of the untreated castrate. Alkaline phosphatase concentration as measured by its activity per gm. dry weight of tissue varied during the 20 days of hormone treatment (Figure 1); this activity was greatest by the fifth day when the average wet weight of the gland was 203 mg. and after this time there was a decrease in activity which was associated with the increase in the size of the gland which was 525 mg. after 20 days of injection (Table 3). Due to the greater relative increase in the various constituents of the gland, the absolute activity of this enzyme per unit of weight becomes less from the fifth to the twentieth day of injection. The alkaline phosphatase of seminal vesicle tissue may be involved in the synthesis of biological phosphate esters as has been demon-

strated for alkaline intestinal phosphatase by Meyerhof and Green (1950). However, the concentration of acid phosphatase was maximal by 10 days of injection and this was followed by a slight decrease by the end of the 20 days (Figure 1). Stafford, Rubinstein, and Meyer (1949) have demon-

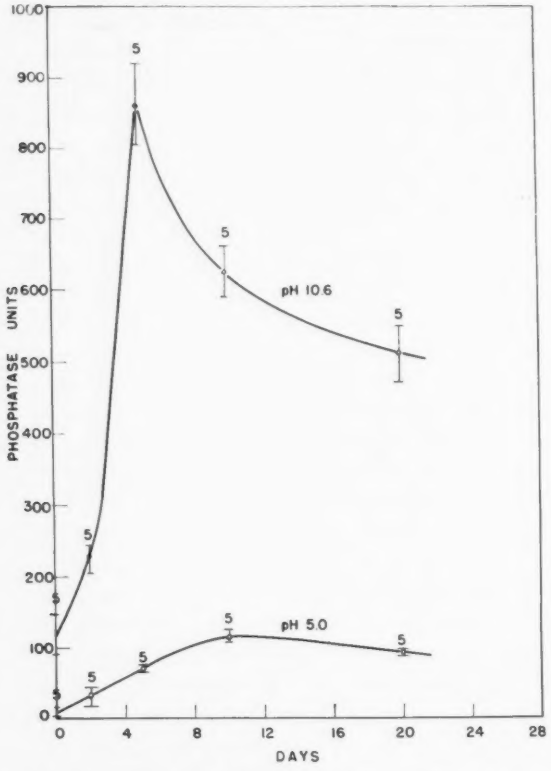

FIG. 1. Effect of testosterone propionate (500 micrograms per day) on the phosphatase activity of secretion-free seminal vesicles of castrate rats. Data are presented as means with standard errors. The number of determinations are indicated above each mean. Legend to curves: upper curve, alkaline phosphatase; lower curve, acid phosphatase.

strated that in castrate rats the administration of 600 μg. of testosterone propionate per day in 2 daily doses for 8 days caused hypertrophy of the seminal vesicles beyond normal size, but under the conditions of the experiments, the acid and alkaline phosphatase activity was not increased beyond normal levels.

The importance of androgen to protein synthesis in the seminal vesicles

is further illustrated by considering the various interrelationships of the data. The primary function of the seminal vesicles is the production of secretion and the most abundant constituent is protein as indicated by its 13.6 per cent nitrogen. Furthermore, the various investigations of Kochakian (1947) have demonstrated the importance of different androgens in protein anabolism in various tissues such as the accessory sex organs, skeletal muscles, and kidneys.

It is to be noted from these data that during the first 2 days of hormone injection, there is a significant increase in the Q_{O_2}. At this time there are only small increases in the catheptic, alkaline and acid phosphatase activities, and nitrogen content of the gland. However, from the second to the fifth day of hormone treatment there is a significant decrease in the Q_{O_2} which is accompanied by an increase in catheptic and phosphatase activity, particularly alkaline phosphatase, and the nitrogen content of the gland. The marked accumulation of secretion appears after the fifth day of hormone treatment.

SUMMARY

In the normal development of the seminal vesicles of the rat, it was found that the increase in the dry weight of the secretion-free gland was a linear function of the body weight in animals weighing approximately 150 gm. or more. The total nitrogen content of the seminal vesicles of prepubertal rats was 0.3 mg.; whereas, it increased to 13.6 mg. in rats weighing 385 gm. The lipid content increased from 0.3 mg. in rats weighing 146 gm. to 4.4 mg. in rats weighing 385 gm. The composition of the seminal-vesicle secretion on a percentage basis was as follows: 25.9 dry weight; 13.6 nitrogen; 1.6 ash; 0.44 non-protein nitrogen; and 0.35 total reducing substances. This secretion contained no lipid.

Castration caused the weight of the seminal vesicles to decrease 73 per cent after 20 days. The catheptic activity of these glands changed little during the first 3 days after castration; however, this activity gradually decreased to 0.9 of a unit after 24 days in contrast to 2.8 units at the time of castration. The total nitrogen content per gland decreased 74 per cent by the twentieth day after castration. Activities of the alkaline and acid phosphatases per gland decreased 81 and 65 per cent, respectively. The Q_{O_2} of seminal-vesicle tissue was not altered by castration.

Injection of testosterone propionate (500 μg. per day) into castrate rats for 20 days resulted in the following increases per gland: wet weight, 48 to 525 mg.; nitrogen, 0.2 to 7.3 mg.; secretion, 0 to 1322 mg.; alkaline phosphatase, 1.3 to 62.8 units; and acid phosphatase, 0.1 to 11.5 units. The catheptic activity of rats that were injected over a 10 day period changed from 0.9 to 4.5 units. From the second to the fifth day of injection the

catheptic activity per gland and the total nitrogen increased 200 and 275 per cent, respectively, and the alkaline phosphatase activity per gland showed a 1061 per cent gain. Acid phosphatase activity increased 520 per cent during the same time. The Q_{O_2} of rat seminal-vesicle tissue was significantly greater 2 days after hormone treatment than that of castrates. However, this rise decreased after this time and by 20 days of treatment there was no significant difference between the Q_{O_2} of seminal-vesicle tissue of injected and uninjected castrates.

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THE EFFECT OF THE ADMINISTRATION OF ESTROGENS ON THE RENIN-SUBSTRATE (HYPERTENSINOGEN) CONTENT OF RAT PLASMA

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LEVIN (1945) pointed out that diethylstilbestrol acts as an adrenocorticotrophic substance as judged by several criteria. For instance, he found that the administration of this estrogen to rats caused a marked lowering of the cholesterol content of the adrenal glands. Since ACTH causes an elevation of the renin-substrate of the plasma of dogs (Haynes Forsham, Thorn, and Dexter, 1949) and rats (Helmer and Griffith, 1951), diethylstilbestrol was administered to rats in order to determine whether it would, like ACTH, cause an increase in the renin-substrate content of the plasma. In addition to stilbestrol, several other estrogens and stilbene compounds were studied.

METHODS

Male rats of the Long-Evans strain weighing 200 to 400 gm. were used. These included normal, adrenalectomized, and hypophysectomized groups. The compounds tested were dissolved in sesame oil and injected subcutaneously. Care of the animals, methods of obtaining plasma samples, and assay of renin-substrate have been described previously in detail (Helmer and Griffith, 1951) and are only briefly summarized here.

Renin-substrate was determined by incubating 1 ml. of heparinized plasma with an excess of hog renin for 5 minutes at 37° C. The reaction was stopped by boiling, the coagulated protein removed by centrifugation, and the resulting clear solution assayed for angiotonin activity. The renin-substrate is expressed as units of angiotonin formed by 1 ml. of plasma.

RESULTS

The Effect of Diethylstilbestrol and Other Estrogens on Normal Rats

Subcutaneous administration of 0.5 mg. of diethylstilbestrol per day caused a marked increase in the renin-substrate content of the plasma of 6 normal rats (Figure 1). After the sharp initial rise the renin-substrate continued to increase gradually until by the 45th day values approximately three times normal were observed. As a result of this treatment the weight of the rats decreased from an average of 389 gm. to 312 gm.

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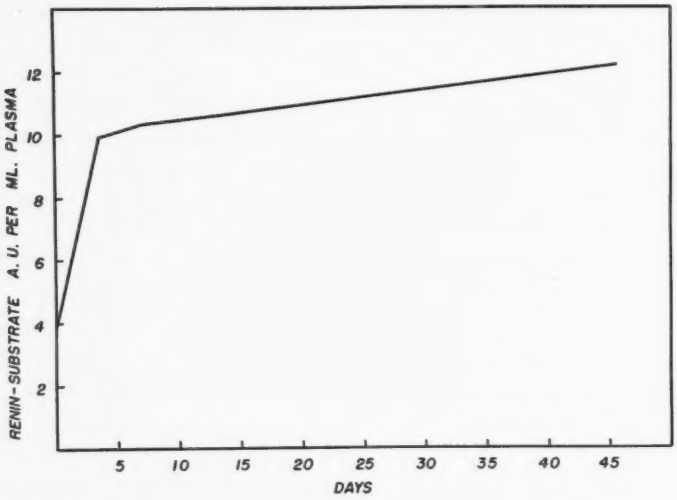

FIG. 1. The effect of the daily subcutaneous injection of 0.5 mg. of diethylstilbestrol on the renin-substrate of normal rats.

TABLE 1. THE EFFECT OF ESTROGENS ON THE RENIN-SUBSTRATE OF NORMAL RATS

Compound tested	No. of rats	Dose mg./ day	Angiotonin units/ml. plasma			Relative activity of compound
			Control	7 days	Change	
Diethylstilbestrol	4	0.01	5.58	7.38	1.80	100
	8	0.02	4.24	7.72	3.48	
	4	0.05	3.99	9.58	5.59	
	4	0.166	4.43	13.21	8.78	
7-Methoxy dl-α-bisdehydrodio-synolic acid	4	0.005	3.67	6.50	2.83	300
Dihydrodiethyl stilbestrol	4	0.02	3.91	8.79	4.88	190
Ethinyl estradiol	4	0.02	3.22	7.74	4.52	160
Hexestrol dimethyl ether	4	0.20	3.51	9.93	6.42	32
Diethyl stilbestrol dimethyl ether	4	0.20	3.25	8.91	5.66	25
Benzestrol	4	0.166	4.25	8.09	3.84	14.5
Estradiol benzoate	3	0.166	4.81	7.33	2.52	8.4
dl-Equilenin	4	0.50	4.08	6.39	2.31	2.4
Estrone	4	0.50	4.11	6.24	2.13	2
4,4′-Di-(4″-methyl 2″-glyoxalyl)-stilbene	4	0.50	4.23	4.51	0.28	0.09
Diamidinostilbene dihydrochloride	4	0.50	3.97	4.21	0.24	0.08
4,4′-Dihydroxy stilbene	4	0.50	3.69	3.72	0.03	0
4,4′-Diamidino stilbene diacetate	4	0.50	4.30	4.29	−0.01	0
4,4′-Stilbene dicarboxylic acid	4	0.50	4.86	4.82	−0.04	0
3,5-Dimethoxy stilbene	4	0.50	4.56	4.47	−0.09	0
4,4′-Dinitro stilbene	4	0.50	3.68	3.61	−0.07	0
1,2-bis-(p-aminophenyl)-2-methyl propanone-1-dihydrochloride	4	0.50	4.03	3.93	−0.10	0
p-Acetylaminobenzaldehyde thiosemicarbazone	4	2.00	5.03	4.90	−0.13	0
2,4′-Dicyanostilbene	4	0.50	4.65	4.29	−0.36	0

by the 14th day. An additional 25 gm. was lost during the remainder of the test.

In order to determine whether the ability to elevate the renin-substrate was a common property of estrogens, a group of synthetic and natural estrogens as well as stilbene derivatives were assayed. Their relative activity was compared to that of stilbestrol. In order to establish a curve with which the activity of the other estrogens could be compared, a series of experiments was performed in which daily doses of stilbestrol ranging from 0.01 to 0.166 mg. were administered over a period of 7 days. These data are shown in the upper part of Table 1, and it will be noted that the synthetic estrogens were the more active compounds. The most active compound tested was 7-methoxy-dl-α-bisdehydrodiosynolic acid.

TABLE 2. EFFECT OF DIETHYLSTILBESTROL ON THE RENIN-SUBSTRATE OF HYPOPHYSECTOMIZED AND ADRENALECTOMIZED RATS

Type of rat	No. of rats	Dose mg./day	Angiotonin units per ml. plasma			
			Control	Days on compound 7	14	Change
Normal	6	0.5	3.91		11.44	7.53
Hypophysectomized	6	0.5	2.26		4.31	2.05
Normal	2	0.5	3.60	10.64		7.04
Adrenalectomized	4	0.5	1.29	6.52		5.23
Normal	8	0.02	4.24	7.72		3.48
Adrenalectomized	4	0.02	2.14	3.98		1.84

The Effect of Diethylstilbestrol on Hypophysectomized and Adrenalectomized Rats

Since stilbestrol was so effective in raising the renin-substrate in normal rats, a series of hypophysectomized and adrenalectomized rats was treated with this compound in order to determine whether this action was mediated through the pituitary-adrenal system.

As shown in Table 2, the response in the hypophysectomized rats was considerably less than in the normal animals. With doses of 0.5 mg. per day for 7 days, stilbestrol was only slightly less effective in the adrenalectomized rats than in normal ones. Since in this instance the dosage was probably too high to demonstrate maximum differences, the experiment was repeated using 0.02 mg. of stilbestrol per day. With the reduced dosage the elevation in renin-substrate in the adrenalectomized rats was approximately one-half that of the normal animals. Apparently, as far as renin-substrate production is concerned, the action of diethylstilbestrol is not necessarily mediated through the pituitary-adrenal system but is in part direct.

The Inhibiting Effect of Testosterone on Diethylstilbestrol

Since some of the physiological actions of estrogens are inhibited by testosterone and progesterone, experiments were designed as shown in Table 3 to see if the response of renin-substrate to stilbestrol was affected by these compounds. Four milligrams of testosterone propionate reduced by 69 per cent the elevation produced by 0.02 mg. of stilbestrol. In addition to inhibiting by 45 per cent the renin-substrate elevation caused by 0.166 mg. of stilbestrol, 8 mg. of testosterone propionate definitely reduced the weight loss that accompanies stilbestrol administration.

TABLE 3. THE EFFECT OF TESTOSTERONE OR PROGESTERONE ON THE ELEVATION OF RENIN-SUBSTRATE PRODUCED BY STILBESTROL OR ESTRONE

Compounds and dosage, mg.		No. of rats	Angiotonin units/ml. plasma			Per cent inhibition	Ratio compound: estrogen	Change in weight, gm.
			Control	7 days on compound	Change			
Stilbestrol	0.02	4	3.93	7.68	3.75			−35.0
Testosterone	2.0	4	4.32	4.27	−0.05			− 2.5
Stilbestrol + Testosterone	0.02 + 4.0	4	4.16	5.32	1.16	69	200:1	−25.0
Progesterone	2.0	4	4.48	4.60	0.12			+ 2.5
Stilbestrol + Progesterone	0.02 + 4.0	4	4.09	7.70	3.61	0	200:1	−55.0
Stilbestrol	0.166	4	4.43	13.21	8.78			−62.5
Testosterone	8.0	4	3.68	4.84	1.16			− 6.5
Stilbestrol + Testosterone	0.166 + 8.0	4	4.17	9.01	4.84	45	47:1	−24.0
Estrone	0.5	4	4.11	6.23	2.12			−31.0
Estrone + Testosterone	0.5 + 4.0	4	3.49	6.11	2.62	0	8:1	−11.0

Against 0.5 mg. of estrone, 4 mg. of testosterone propionate was ineffective. In the doses used progesterone had no inhibiting effect on stilbestrol.

The Effect of Diethylstilbestrol on Man

A male patient with carcinoma of the prostate gland was given 25 mg. of diethylstilbestrol for 7 days. The renin-substrate concentration of the plasma, which was 2.64 angiotonin units per ml. before the start of the therapy, rose to 9.25 angiotonin units per ml.

The plasma renin-substrate of a male patient with arthritis who was subjected to the same regimen rose from 2.64 to 10 angiotonin units per ml. There was no alleviation of the arthritic symptoms.

DISCUSSION

Diethylstilbestrol, which, like ACTH, lowers the cholesterol content of the adrenal gland, also causes an increase in the renin-substrate concentration of the plasma. As a matter of fact it causes a greater increase than ACTH, 0.02 mg. of stilbestrol being more effective than 5 mg. of ACTH.

Since the response of the adrenalectomized rat to stilbestrol was about one-half that of the intact rat, it appears that this estrogen exerts some effect on renin-substrate production which is independent of the pituitary-adrenal system. These results are similar to those reported by Ingle (1943), who, in studying the relationship of the diabetogenic effect of diethylstilbestrol on the adrenal cortex of the rat, found that it exerted a diabetogenic effect not mediated through the adrenal cortex.

Since the administration of stilbestrol causes a marked loss of body weight in animals, the accompanying hypertrophy and lowered cholesterol content of the adrenal gland may be the result of a stressful situation. The amount of adrenal corticoid produced may be just sufficient to meet the increased demands of the stress and may not contribute to the renin-substrate elevation. It is also possible that the reduction of the renin-substrate response to stilbestrol in the adrenalectomized rats may be due to the lowering of the metabolic activity of the tissues which synthesize renin-substrate. If this is so, the action of stilbestrol on renin-substrate may be independent of the pituitary-adrenal system.

The failure of the estrogens as efficacious agents for the treatment of rheumatoid arthritis (Clark, 1949) further indicates that these compounds may not cause the production of significant amounts of corticosteroids beyond that needed to meet the stress. In this connection it is of interest to note the four-fold increase in renin-substrate found in the plasma of the patient suffering from arthritis who was treated with 25 mg. diethylstilbestrol for 7 days without alleviation of the symptoms of arthritis.

Robson (1938) found that testosterone propionate inhibited the vaginal cornifying action of estrone in spayed rats when testosterone propionate and estrone were injected in the ratio 250:1. The antagonistic effect of testosterone propionate on the renin-substrate response to diethylstilbestrol in rats is of the same order (200:1). The data in this paper also agree with the findings of Robson that progesterone is a less effective inhibitor of the estrogens than testosterone proprionate.

Although 0.5 mg. of estrone does not elevate plasma renin-substrate to the same degree as 0.02 mg. of stilbestrol, the amount of testosterone propionate (4 mg.) that would inhibit 0.02 mg. of stilbestrol showed no antagonism to 0.5 mg. of estrone. This indicates that the antagonism is based on a molecular ratio of the compounds in question rather than on the physiological responses.

The ability to increase renin-substrate appears to be a common property of the group of estrogens tested. The estimation of the plasma renin-substrate of rats after the administration of these compounds offers another means of testing their biological activity.

SUMMARY

The administration of diethylstilbestrol to normal male rats caused a marked elevation in the renin-substrate concentration of the plasma. This effect is not necessarily mediated through the pituitary-adrenal system, although in the absence of the adrenal glands the response is reduced.

Testosterone propionate inhibits the renin-substrate response to diethylstilbestrol.

A test for the biological activity of estrogens based on the determination of the renin-substrate content of the plasma of normal rats is presented.

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THE ROLE OF THE GASTROINTESTINAL TRACT, INCLUDING THE LIVER, IN THE METABOLISM OF RADIOTHYROXINE*

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INTRODUCTION

THE role of the gastrointestinal tract, including the liver, in the metabolism of thyroid hormone has been debated for years. Older experiments on biliary excretion and gastrointestinal reabsorption of thyroidal substances have been summarized by Elmer (1938), and more recent studies, some with more refined and others with newer technics, by Monroe and Turner (1949). From these studies has arisen the concept that the "liver rids the body of excess thyroxine." Certain doubts concerning the validity of this idea have been raised by recent observations with minute amounts of radioactive thyroxine and other radioactive thyroidal materials in the human and the rat (Hamilton *et al.*, 1949; Albert and Keating, 1949; Albert *et al.*, 1949; Albert and Keating, 1951). Considerable radioactivity was constantly found to be localized in the region of the liver in the human. Direct analysis of tissues in the rat disclosed that the liver and gastrointestinal tract concentrated large amounts of I^{131} after the injection of radiothyroxine (Gross and Leblond, 1947). Sequential studies further indicated that radioactive material was transferred from the liver to the gastrointestinal tract mainly by way of the bile and thence along the gastrointestinal tract (Johnson and Albert, 1951). Considerable reabsorption could therefore occur, and only part of the radioactive material reaching the gastrointestinal tract would be eventually excreted in the feces.

The purpose of this study was (1) to assess the magnitude of the enterohepatic circulation of thyroid hormone and (2) to determine whether this circulation represented a detoxication process or was a normally occurring phase in the intermediary metabolism of thyroid hormone.

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MATERIAL AND METHODS

Male weanling rats of the Wistar strain were used for all experiments. They were about 1 month of age and ranged in weight from 35 to 55 gm. The animals were maintained on ordinary laboratory diet and tap water. In the first experiment 52 rats were employed. Each rat was lightly anesthetized, and radioactive l-thyroxine was injected into the external jugular vein. When a group of 4 rats had been thus injected, the rats were placed in glass metabolic cages for collection of combined urine and feces. At varying intervals after injection, a group of 4 rats was killed, and the radioactivity in the combined urine and feces, the liver, stomach, small intestine and large intestine, and also in the remaining portion of each carcass, was determined. Thirty-two animals were injected with 0.6 microgram of radioactive l-thyroxine[1] labeled with 2 microcuries of I^{131}. The volume of injection was 0.25 cc. and the animals were killed at 1, 15, 30, 45 and 60 minutes and at 6, 12 and 24 hours after injection. For the remaining 20 animals the dose was 0.25 cc. containing 2.5 micrograms of l-thyroxine labeled with 5 microcuries of I^{131}. These animals were killed at daily intervals for 5 days.

This experiment was repeated in greater detail with several modifications, employing a total of 60 rats. Twenty-eight animals were given 7 micrograms of l-thyroxine labeled with 2 microcuries of I^{131}. Groups of 4 rats each were killed after 1, 30 and 60 minutes and after 3, 6, 12 and 24 hours. Thirty-two rats were also given 7 micrograms of l-thyroxine, labeled with 6 microcuries of I^{131} and were killed after 2, 3, 4, 5, 7, 9, 11 and 16 days. Thus the same quantity of thyroxine was administered to all rats but the amount of radiation was adjusted for convenience of counting. The urine and feces were collected separately and measured. Animals were weighed before necropsy, and the weights of all tissues removed for counting were recorded. Blood was collected from each rat, pooled and counted. In addition to liver, stomach, small and large intestines, the kidneys were removed. On several occasions the thyroid was dissected from the carcass and its I^{131} was separately determined.

Radioiodine was measured according to a method previously described (Johnson and Albert, 1951). It was found unnecessary to compare each experiment with a pilot of the dose. The sum of the counts recorded in the organs removed and the residual carcass was found to equal, within a few counts, the total *in vivo* count of the unanesthetized rat before necropsy. At any interval following injection, the sum of the counts in feces, urine, organs and carcass also equaled, with due correction for physical decay, the immediate, postinjection *in vivo* count of the entire living rat. When the sum of all counts for each interval was plotted on a semilogarithmic scale, a curve resulted which had a half-value time of exactly 8.0 days, the physical half-life of I^{131}. Fitting by the method of least squares gave a standard deviation of the observations of 3.2 per cent.[2] This figure provides a measure of all errors resulting from loss of material on beakers, syringes, during injections or via urine and feces in metabolic cages, as well as technical errors in counting.

RESULTS

The results of the first experiment are shown in Figure 1. One minute after completion of injection, 43 per cent of injected radioactivity was in

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[1] Radio-l-thyroxine was obtained from Abbott Laboratories and was stated to be chemically pure as monitored by paper chromatography.

[2] We are indebted to Dr. Joseph Berkson for statistical analysis of this curve.

the liver. This value fell rapidly to 25 per cent after 45 minutes and 6.9 per cent at the end of 24 hours. The stomach, small bowel and large bowel had progressively increasing amounts of I^{131} during the 6 hours following injection. The sum of these values (32 per cent of the dose) has the same order of magnitude as the reduction in radioactivity (28 per cent) which had taken place in the liver during the same 6-hour interval. Excretion

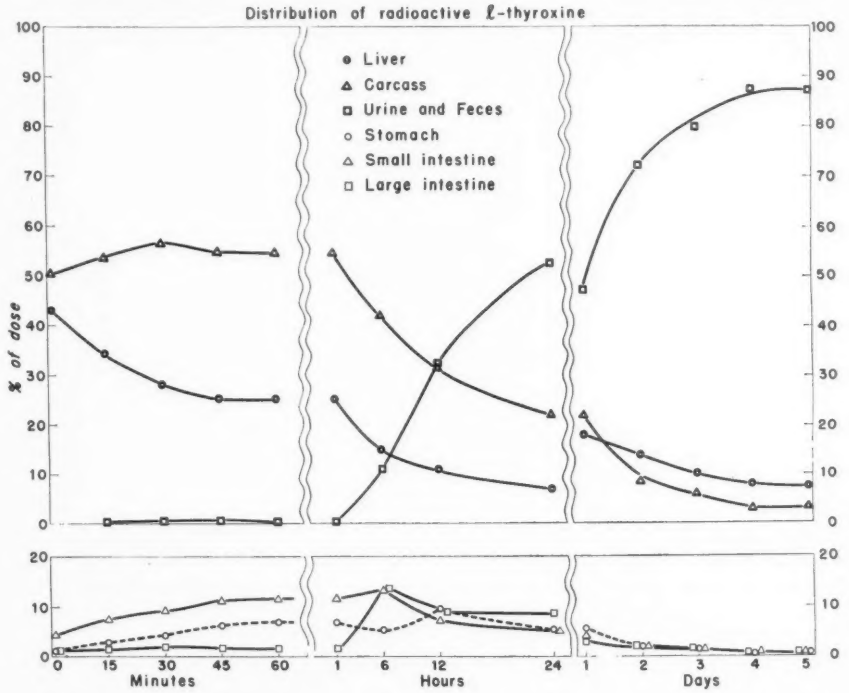

Fig. 1. Distribution of radioactivity following a single intravenous injection of radioactive l-thyroxine. Note that the abscissa is separated into three phases showing events occurring during minutes, hours and days following administration of thyroid hormone. Each point represents mean values obtained from 4 rats.

via urine and feces began within 6 hours, and increased throughout the 5 days, at the end of which time 87 per cent of the dose had been excreted. Of the radioactivity remaining in the body, an increasing proportion accumulated in the liver. After about 36 hours, the liver contained more I^{131} than the rest of the carcass; and at the end of 5 days, the liver contained 59 per cent of the remaining radioactivity while the carcass, exclusive of gastrointestinal tract, contained 26 per cent.

In order to elucidate several aspects of the foregoing findings, a second

TABLE 1. DISTRIBUTION OF I^{131} FOLLOWING INTRAVENOUS INJECTION OF RADIO-1-THYROXINE

Organ*	Hours														
	1/60	½	1	3	6	12	24	42	60	84	108	156	228	300	377
	Per cent of dose														
Blood†	4.23	2.66	2.07	1.35	1.03	—	0.36	0.158	0.097	0.075	0.03	0.014	0.0046	0.0029	0.0044
Stomach	1.09	3.66	4.41	4.40	4.95	5.82	3.30	1.29	0.63	0.55	0.17	0.40	0.16	0.11	0.08
Small intestine	5.11	19.58	23.13	30.90	14.55	7.26	3.89	1.26	0.66	0.52	0.26				
Large intestine	1.26	1.31	1.25	1.91	19.57	7.15	3.47	1.82	0.79	0.86	0.39				
Liver	30.26	19.98	16.57	11.66	8.75	6.28	3.34	5.27	3.75	3.02	2.42	1.90	1.51	1.32	1.18
Kidneys	5.97	4.53	3.30	2.46	1.78	1.33	0.72	0.34	0.23	0.15	0.11	0.07	0.08	0.07	0.06
Carcass	38.55	45.14	43.03	40.00	30.58	24.60	12.90	8.17	5.84	5.00	3.37	2.16	1.08	0.95	0.89
Urine	0	0.49	1.86	3.52	8.53	13.91	20.12	29.53	30.60	34.96	32.40	36.83	33.96	35.89	37.22
Feces	0	0.01	0.03	0.02	7.08	30.41	50.77	51.60	57.12	54.61	60.74	58.58	63.16	61.64	60.52

* Each value is the mean of observations on 4 rats.
† Per cent of dose per gram.
All other values are per cent of dose.

experiment was done, in which observations were extended over a period of 16 days. The essential data are summarized in Table 1. In Figure 2 are plotted the curves of excretion of urine and feces, and of residual radioactivity in the body. The relationship of these curves to one another is evident. The radioactivity leaves the body rapidly, about twice as much leaving via the feces as via the urine. The curves for urine and feces both

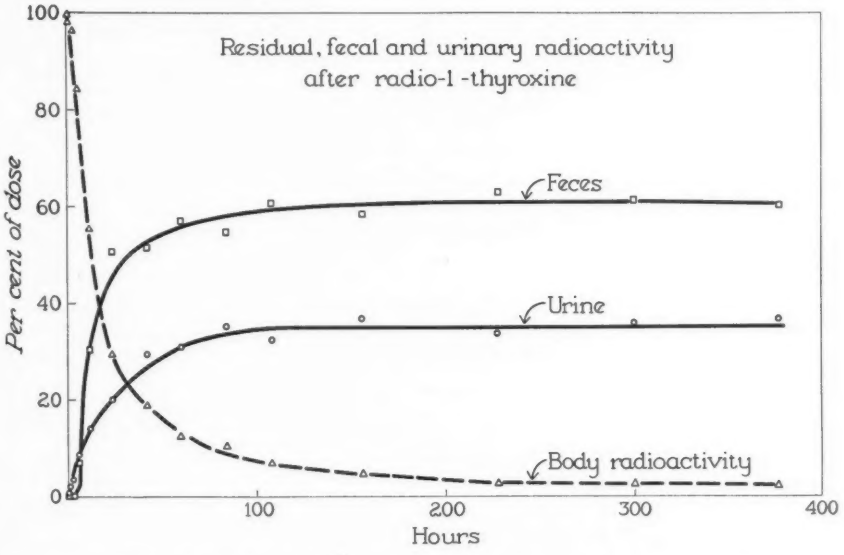

FIG. 2. The relationship between the total body I^{131} and the excretion of I^{131} in urine and feces after a single dose of radio-1-thyroxine.

have similar half-value times. Values for both show some fluctuation, particularly toward the end of the experiment, but when values for urine and feces are combined, these minor fluctuations cancel out and a smooth curve results. The curve for residual radioactivity is the complement of this sum and illustrates this point. Evidently the separation of urine and feces in the metabolism cages was not complete.

The curves for both urine and feces fit a function of the form $(Q = Qf[1 - e^{-rt}])$ where Q is the quantity of radioactivity excreted at any time, t, expressed as percentage of dose, Qf is the asymptotic amount corresponding to $t = \infty$ and r is a rate constant. The value Qf is 31.5 per cent of the dose in the case of urine, and 60 per cent of the dose in the case of feces. The half-value time is 15 hours and the value for r is 0.046 or 4.6 per cent per hour in each case. Using a model analogous to that previously developed for urinary inorganic iodide (Keating *et al.*, 1947), the

proportional rate of fecal excretion is given by Qf (feces) $\times r$ or 2.8 per cent per hour, and the proportional rate of urinary excretion is given by Qf (urine) $\times r$ or 1.4 per cent per hour.

The curve representing residual radioactivity has at least two regressions and can be fitted to the function $Q_r = Q_a e^{-\alpha t} + Q_b e^{-\beta t}$ where Q_r is residual activity at any time, t, expressed as percentage of dose; Q_a and α are the extrapolated value corresponding to $t = 0$ and the rate constant, respectively, for the first component; and Q_b and β are the corresponding values for the second component. They may be estimated by graphic means as described elsewhere (Dominguez *et al.*, 1947; Burch *et al.*, 1949, 1950): $Q_a = 90$ per cent, $\alpha = 0.051$, half-value time, 13.5 hours; $Q_b = 10.1$ per cent, $\beta = 0.0049$, half-value time, 140 hours. These values suggest: (1) that about 90 per cent of the radioactive material is excreted at a rate of 5.1 per cent per hour, and (2) that the remaining 10 per cent is involved in a much slower process, leading to excretion at a rate of 0.49 per cent per hour. This change in the proportional rate of excretion does not imply a real change in excretory function of kidneys and bowel with respect to the injected thyroxine but reflects a change in the effective volume of distribution[3] of thyroxine, or, to express it in another way, a change in the magnitude of the metabolic pool with which the radiothyroxine in the blood is in equilibrium. Values for volume of distribution may be estimated directly for each observation (Myant *et al.*, 1949) or from the blood curve itself: V_1, the volume for the initial portion (estimated from component B of the blood curve, Figure 3), is of the order of 100 ml.; and V_2, the volume for the second portion (estimated from component C of the blood curve), is of the order of 1,200 ml. A reliable measure of total excretion is given by total plasma clearance, which is the product of rate constant and volume of distribution. For the first portion this is $\alpha \times V_1$ (0.051×100) or 5.1 ml. per hour. For the second portion it is $\beta \times V_2$ ($0.0049 \times 1{,}200$) or 5.9 ml. per hour, which is not significantly different from the first value. This fact supports the view that the change in proportional rate does not represent a change in the actual excretion process. The arithmetical relationships involved are discussed elsewhere (Berkson *et al.*, 1950).

At least three regression rates may be estimated from the more complex curve of radioactivity in the blood (Figure 3), which may be fitted to a multiple exponential function analogous to that described in an earlier

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[3] Volume of distribution is here defined as that volume expressed in milliliters which would be occupied by all the radioactivity in the body at any time, assuming a uniform concentration identical with that in blood. As the foregoing values for immature rats indicate, it is an entirely theoretical albeit useful value.

paragraph for residual radioactivity. Component A, comprising the initial phase of the blood curve, during the first 6 hours, has a half-value time estimated at 36 minutes (corresponding to a rate constant of 117 per cent per hour) and evidently, as shown later, represents equilibration of radioactivity with a larger volume of distribution almost entirely within the gastrointestinal tract. Component B, comprising the second major portion of the curve, between 24 and 108 hours, has a half-value time estimated as 15 hours (4.6 per cent per hour). This corresponds closely to the rate

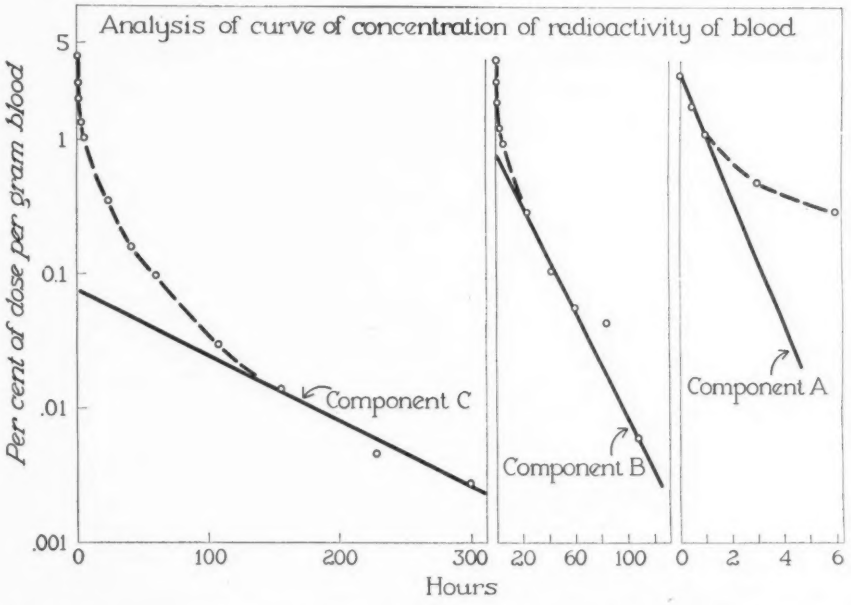

FIG. 3. The curve of concentration of I^{131} in the blood following administration of radiothyroxine has been segregated into three components, derived by graphic means.

constant derived from curves of fecal excretion, urinary excretion and residual radioactivity, and evidently reflects the disappearance of radiothyroxine from its initial volume of distribution into the excreta, as well as into tissues. Component C, comprising the third major component of the curve, between 108 and 377 hours, has a half-value time of 62 hours (1.1 per cent per hour). It appears to be analogous to the second component of the curve of residual radioactivity, but the fact that it is twice as rapid as the second component of the curve of residual radioactivity indicates that I^{131} is leaving blood by some other route than urine and feces into some larger equilibrium volume within the body. A possible

fourth component (indicated by dotted line between components A and B in Figure 3) is not readily analyzed but probably reflects portal reabsorption. It is to be emphasized that such a curve as that depicted in Figure 3 actually represents complex biologic phenomena having an indeterminate number of exponential components; the regression rates described were chosen (1) because of goodness of fit, (2) because they could be correlated with other independent observations, and (3) because they could be in-

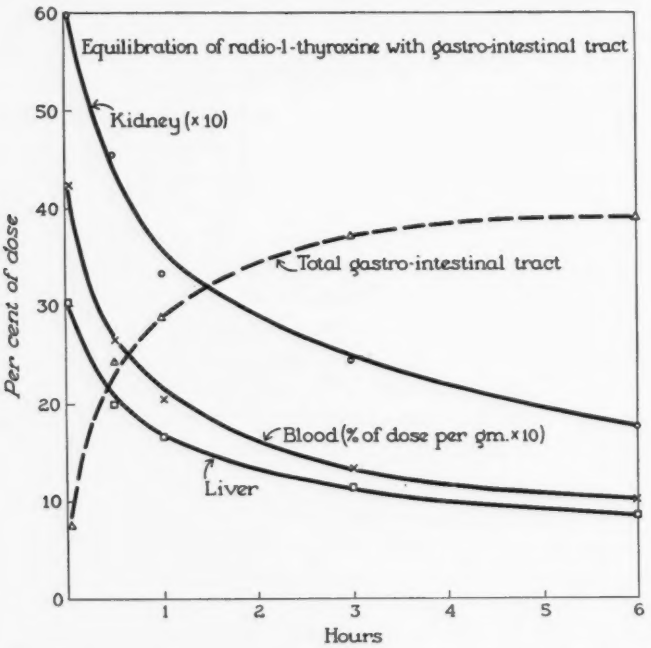

FIG. 4. The relationship between the fall of radioactivity in blood, liver and kidneys and the increase in I^{131} in the entire gastrointestinal tract. Note that the shapes of the curves for kidney, blood and liver are identical and appear inversely related to the curve for gastrointestinal I^{131}.

terpreted in terms of a model describing an orderly and chronologic sequence of kinetic events.

The initial rise of radioactivity in the small bowel, followed after an interval by a similar rise in the large bowel, suggests that most of the I^{131} of the gastrointestinal tract reaches it via the bile. The behavior of I^{131} in the stomach, to which this statement does not apply, can be interpreted, on the basis of the studies of Johnson and Albert (1951), to reflect to a large extent the behavior of inorganic iodide. The relationship between

component A of the blood curve and the gastrointestinal tract is shown in Figure 4, where the distribution of radioactivity during the first 6 hours following injection is plotted. The curves for both liver and kidneys fall at the same rate as that for blood. The values for carcass also diminish during this interval (Table 1). The quantities of I^{131} which disappeared from liver, kidneys, carcass and blood are almost entirely accounted for by material which has entered the gastrointestinal tract. A total of 47 per cent of the dose has shifted from the blood, liver and carcass between the first minute and the sixth hour at which time 32 per cent is in the gastrointestinal tract, and 15 per cent in urine or feces. All four curves shown in Figure 4 evidently reflect the same event, namely, equilibration with the gastrointestinal tract. This is furthermore borne out by calculation of the ratio, $\frac{\text{per cent of dose in gastrointestinal tract}}{\text{per cent of dose/gm. blood}}$. This ratio increased from 1.8 in the first minute to 37.9 at 6 hours. After 6 hours this ratio shows fluctuation but no significant regression, being 37.9 at 300 hours. All four curves have a half-value time of the same order of magnitude as component A of blood, that is, about 30 minutes.

If the radioactivity remaining in the body at any time be taken as 100 per cent, the amount of I^{131} present in any of the organs may be expressed as a proportion of total body radioactivity. When values are plotted in this manner several significant relationships are disclosed, the most interesting of which is a progressive increase of radioactivity in liver (Figure 5). This would appear to constitute a second major phase in the metabolism of thyroxine. After 16 days, the liver contains 52.5 per cent of residual radioactivity; the carcass, 38.2 per cent, and the entire blood volume, 3.2 per cent. The curve of radioactivity in the liver appears to follow an exponential curve having an asymptotic value (Qf) of 53 per cent and a half-value time of 62 hours (1.1 per cent per hour). These values appear related to those estimated from component C of the blood curve and the second component of the curve of residual radioactivity.

Further details of this second phase of thyroxine metabolism can be obtained by expressing radioactivity in the various tissues in terms of the ratio, $\frac{\text{tissue concentration}}{\text{blood concentration}}$. Figure 6 suggests that growth of radioactivity also takes place in kidneys and carcass in a manner similar to that in the liver although the concentrations involved are much smaller. No such growth is evident in the gastrointestinal tract, where, if it occurs, it is probably overshadowed and obscured by events occurring within the lumen.

The kinetic data described provide indirect evidence for a vigorous enterohepatic circulation, with thyroxine entering the gastrointestinal tract via the bile and gastrointestinal secretions and leaving it via the portal circulation, lymphatic drainage and feces. Direct experiments were undertaken to demonstrate biliary secretion and portal reabsorption.

Biliary Secretion.—In 20 rats, ligatures were placed at the esophago-

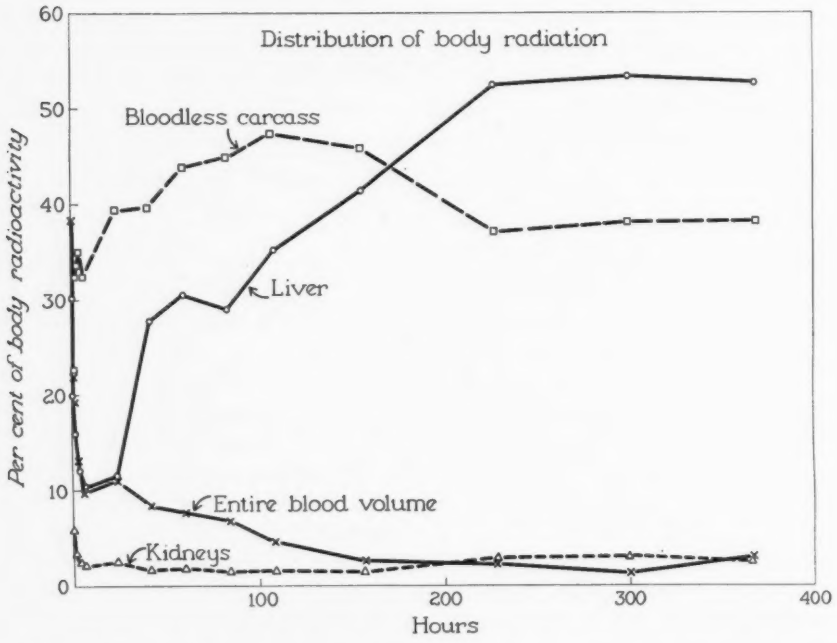

FIG. 5. The distribution of body radiation during the entire experiment shows a marked growth of radioactivity in the liver after an initial drop parallel with that of the blood. The value for entire blood volume was calculated as follows: concentration per gram of blood × blood volume in grams, the latter estimated to be 6.2 per cent of body weight.

gastric juncture, the pylorus, the jejunocolic juncture, and at the anorectal juncture. These ligatures prevented movement of contents along the gastrointestinal tract but did not interfere with blood supply (Albert *et al.*, 1952). In half of the animals the bile ducts were ligated and in the other half, were left intact. Radiothyroxine (2.5 micrograms labeled with 5.0 microcuries I^{131}) was injected intravenously; the animals were killed 4 hours later and the I^{131} was determined as described previously (Table 2). Six to seven times as much radioactivity was present in the

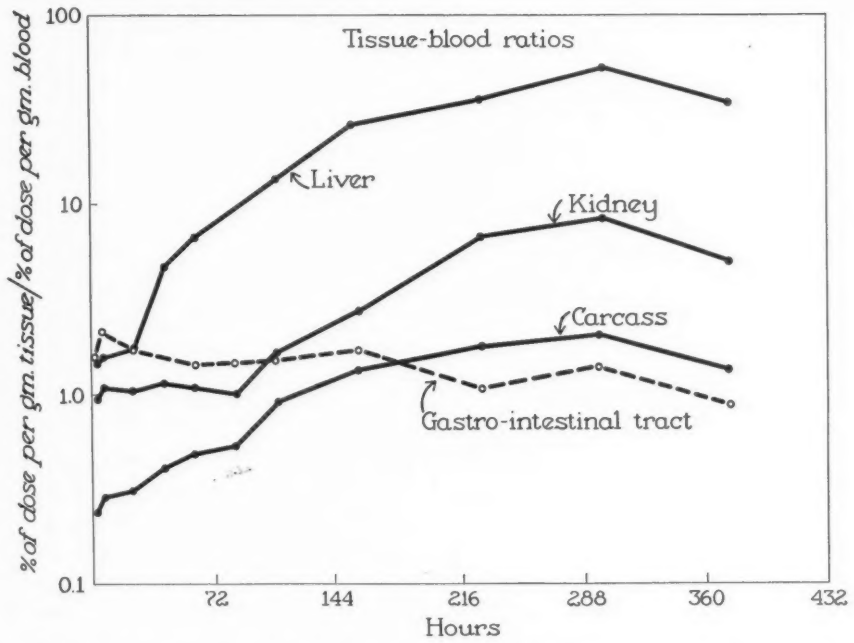

FIG. 6. The concentrating power of liver, kidneys and carcass (tissue-blood ratios) increases progressively for about 8 days, possibly reflecting storage of thyroxine derivatives in tissues. The gastrointestinal tract does not show such growth, possibly because events occurring within the lumen may overshadow and obscure changes in I^{131} of the tissues of the gastrointestinal tract.

small intestine of rats with patent bile ducts as in rats with bile ducts ligated. Thus more than 85 per cent of the I^{131} of the small bowel appears to reach it via the bile, and 15 per cent via direct transfer from blood.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY AFTER INTRAVENOUS ADMINISTRATION OF RADIO-l-THYROXINE IN RATS WITH ISOLATED PORTIONS OF THE GASTROINTESTINAL TRACT

Organ	Mean per cent of dose ± S. E.		"t"
	Bile duct not ligated	Bile duct ligated	
Stomach	5.75 ± 0.25	5.88 ± 0.23	0.38
Small intestine	20.97 ± 1.10	3.55 ± 0.28	14.76
Large intestine	1.04 ± 0.05	1.36 ± 0.07	4.00
Liver	34.98 ± 0.76	39.13 ± 1.16	2.33

In another experiment, 24 rats were injected intravenously with radiothyroxine (0.28 microgram labeled with 0.5 microcurie I^{131}). Half of these had intact bile ducts; in the other half bile ducts were ligated. After injection, animals were placed in metabolic cages for 48 hours, then killed,

dissected and measured as previously described. The results (Table 3) show that fecal excretion in ligated animals was one fifth to one sixth that of normal animals, confirming the proportion of fecal I^{131} excretion contributed by biliary I^{131}.

TABLE 3. EFFECT OF LIGATION OF BILE DUCT ON DISTRIBUTION AND EXCRETION OF I^{131} AFTER INTRAVENOUS ADMINISTRATION OF RADIO-l-THYROXINE

Condition	No.	Per cent of dose						
		St.	S.I.	L.I.	Liver	Carcass	Urine	Feces
Normal	12	2.3	2.2	3.3	3.0	12.2	38.9	37.3
Bile ducts ligated	12	9.4	3.2	2.3	3.5	26.6	47.7	7.4

Abbreviations: St. = Stomach, S.I. = Small Intestine, L.I. = Large Intestine.

Portal Absorption.—Albert *et al.* (1952) had previously found that when thyroxine was injected into the lumen of the isolated small or large bowel 20 per cent or more was reabsorbed and redistributed in various tissues in a pattern similar to that already described. In a similar experiment, radiothyroxine (4.5 micrograms labeled with 0.16 microcurie) was injected into the lumen of the duodenum of 10 animals with intact, and 8 animals with ligated, bile ducts. Excreta were collected and distribution was determined after 48 hours as previously described (Table 4). In the normal animals two thirds of the I^{131} was excreted in the feces and one third was absorbed. Without the function of the biliary system, however, one third was excreted and two thirds retained. Thus it must be assumed that at least one half of the thyroxine instilled in the duodenum of normal animals must have been not only absorbed but recirculated through the liver before appearing in the feces. Portal and arterial blood was withdrawn simultaneously at 3.3 hours after duodenal instillation of radiothyroxine. The liver was also removed and counted. The portal blood contained 65.7 c./s./gm.; arterial blood, 64.2 c./s./gm., and the liver, 121.5 c./s./gm.

TABLE 4. EFFECT OF LIGATION OF BILE DUCT ON DISTRIBUTION AND EXCRETION OF I^{131} AFTER INTRADUODENAL INSTILLATION OF RADIO-l-THYROXINE

Condition	No.	Per cent of dose						
		St.	S.I.	L.I.	Liver	Carcass	Urine	Feces
Normal	10	1.9	1.3	1.0	1.1	5.9	21.2	67.6
Bile ducts ligated	8	8.6	3.4	5.8	2.1	16.9	29.0	34.3

The possibility that the bile may contain thyroxine in different chemical form (Taurog *et al.*, 1951, 1952) or intermediates of thyroxine led us to repeat these experiments with radioactive bile. Large rats (200 to 300 gm.) were injected with 10 to 50 microcuries of radio-1-thyroxine after insertion of a permanent polythene cannula into the bile ducts. Bile was collected for about 6 hours, during which time maximal concentration

of I^{131} was reached. This radioactive bile (0.5 microcurie) was then injected into the duodenum of 9 small rats with, and 10 small rats without, ligation of the bile ducts, and determinations were made 48 hours later (Table 5). Again, only part of the I^{131} instilled in the duodenum was excreted in the feces. With bile ducts intact, part of the radioactive bile was not only absorbed, but resecreted in the bile, and thence in the feces.

TABLE 5. EFFECT OF LIGATION OF BILE DUCT ON DISTRIBUTION AND EXCRETION OF I^{131} AFTER INTRADUODENAL INSTILLATION OF RADIOACTIVE BILE

Condition	No.	St.	S.I.	L.I.	Liver	Carcass	Urine	Feces
Normal	5	0.5	0.3	0.4	0.3	1.7	10.6	86.3
Bile duct ligated	5	5.0	1.6	5.5	1.2	14.0	15.3	57.4
Normal	5	2.6	1.3	1.1	1.1	6.5	14.9	72.6
Bile duct ligated	4	6.8	2.0	2.2	2.3	14.9	27.6	44.1

Direct experiments such as those described in previous paragraphs confirm the presence of an enterohepatic circulation for thyroxine, but, considering the possible effects of surgical trauma and anesthesia on biliary and gastrointestinal function, may not provide a reliable measure of its magnitude. The over-all magnitude of the enterohepatic circulation may be inferred from the rate constants of the curves (Figure 4) representing equilibration between the blood and the gut. More than 100 per cent of all of the circulating thyroxine enters the bowel each hour. The rate of fecal excretion, on the other hand, is of the order of 3 per cent per hour, suggesting a rapid turnover of material from the liver to bile, to gut, to liver.

DISCUSSION

Since only total radioactivity was determined in these studies, one can only conjecture about the chemical state of the I^{131} measured. Previous studies have suggested that radiothyroxine is metabolized to at least two substances, diiodotyrosine and inorganic iodide, neither of which is selectively concentrated in the liver or bile, and both of which disappear from the body much more rapidly than does thyroxine. Consequently, neither diiodotyrosine nor inorganic iodide would occur in sufficient concentration in the liver to distort the present data seriously or to modify their interpretation. Measurable amounts of radioiodide were undoubtedly present in stomach, kidneys and carcass during the first 48 hours and may also have affected somewhat the form of the blood curve during this interval.

The contribution of endogenous radioactive thyroid hormone from the animal's own thyroid may be regarded as negligible since this strain of rats has a poor thyroidal collection of radioiodide. Check of thyroidal radioactivity at several points during the study showed that it never exceeded 2 per cent of residual body radioactivity, a negligible amount.

It has been generally considered that the liver detoxicates and excretes

excessive quantities of thyroxine just as it fulfills a similar role for many other substances. Taurog *et al.* (1951) agreed with this concept and furthermore assumed that the biliary secretion of thyroxine following intravenous injection represents excretion of thyroxine from the body, since in the rat the same proportion of the dose was excreted via a biliary fistula as was normally excreted in feces over the same period. The data here presented cannot be reconciled with such an interpretation. That the amounts of fecal and biliary I^{131} were equal in the experiments of Taurog *et al.* does not preclude the possibility that a rapid turnover of biliary radioactivity had occurred within 24 hours and that it recirculated many times through the liver before appearing eventually in the feces.

The concept that the liver "rids the body of excess thyroxine" carries with it the implication that any single injection of thyroxine, however small, constitutes an excess. This point of issue no longer revolves about the terms "pharmacologic" versus "physiologic." A physiologic dose of thyroxine comparable to the daily output of the rat thyroid has already been studied on many occasions when appropriately labeled with I^{131}. Although such doses are in the physiologic range, it can be alleged that even such small amounts of hormone, injected all at once, produce a temporary excess, whereas under natural conditions a comparable amount is secreted continuously over the period of 24 hours. If such a distinction actually exists in nature, one might expect a large proportion of the dose to be handled initially as an excess. Later, after thorough mixing, some remaining portion of the dose would be handled in the same manner as endogenous hormone. That such may not be the case is suggested by calculating total plasma clearance

$$\frac{\text{(per cent of dose excreted in urine and feces per hour)}}{\text{mean concentration in blood}}.$$

For the first 24 hours this value is 3.6 ml. per hour; for the second 24 hours, 3.8 ml. per hour. For the period from the ninth to the tenth day, it is 4.7; for a period from the thirteenth to the sixteenth day, it is 3.9. These values agree satisfactorily with those obtained by integration of the curve of residual radioactivity and estimates of volume of distribution. Thus rate of excretion of the small portion of radioactivity remaining after 16 days of mixing is of the same order of magnitude as that of the large initial "excess." The constant value for plasma clearance thus refutes the argument of initial hormone excess.[4]

[4] This argument requires the assumption that free thyroxine itself is the circulating hormone (Taurog and Chaikoff, 1949). Neither the constancy of plasma clearance nor any other part of this study bears directly on the question of identity of radiothyroxine and endogenous hormone.

The role of the liver in the enterohepatic circulation of thyroxine is evidently not a simple one, although the initial phase of this role is now clear. One minute after injection nearly as much of the labeled thyroxine was in the liver (30 per cent) as in the entire blood volume (38 per cent). The rest of the carcass, exclusive of the gastrointestinal tract and kidneys, contained only 22.5 per cent at this time. Apparently, circulating thyroxine must enter the liver as freely and rapidly as it mixes with the blood. The values for gastrointestinal tract, kidneys, and carcass also suggest rapid extravascular diffusion but on a smaller scale. For the first few hours after injection the ratio of liver to blood concentration remains essentially the same, despite the events which are taking place in the rest of the gastrointestinal tract. Apparently, as thyroxine leaves the liver via the bile to enter the gastrointestinal tract, it is replenished from the blood and indirectly from other tissues, so that liver, carcass, kidneys and blood all reflect equilibration with the gastrointestinal tract.

The later hepatic phase of thyroxine metabolism is not so clear and any discussion of it is highly tentative. The results shown in Figures 5 and 6 represent, to the best of our knowledge, new findings. They indicate that liver I^{131} follows a triphasic pattern: (1) almost instantaneous concentration, (2) decline due to equilibration with gastrointestinal tract, and (3) a slow regrowth. This slow growth of radioactivity in the third phase is a proportionate one, not an actual increase, for in terms of administered dose, the liver, as well as all tissues, contains progressively less and less I^{131}. However, so far as the constantly dwindling radioactive tag continues to label endogenous similar material in blood and tissues, the growth depicted by Figures 5 and 6 may be presumed to reflect a real event. In proportion to the total I^{131} in the body at any time, there is an accumulation of I^{131} (and inferentially, of I^{127}) in the liver relatively great in proportion to the weight of the liver or to the concentration of I^{131} in the blood. The liver, constituting about 4 per cent of the body weight, contains more than one half of the total I^{131} in the body after 16 days.

A similar slow accumulation of radioactivity also occurs in the kidneys and in the rest of the carcass as well, but in comparatively minute amounts compared with the liver. The curves for liver, kidneys and carcass have comparable slopes. If it is correct to assume that the event depicted is equilibration between blood and some derivative of thyroxine bound in tissues, this slope represents an index of the equilibration rate. It is interesting that the half-value time of this event (62 hours) is of the same order of magnitude as that of the calorigenic action of thyroxine.

Recent work by Taurog *et al.* (1952) suggests that the material secreted in the bile after intravenous injection of l-thyroxine may be thyroxine glucuronide, but that this conjugated form is modified prior to fecal

excretion. It may be of value to learn what, if any, transformations occur during reabsorption from the gastrointestinal tract. The slow phase of liver and tissue thyroxine can be presumed not to be free thyroxine, since it is evidently not in free equilibrium with the blood and hence is different from the initial rapid phase. A recent study by Gross and Pitt-Rivers (1952) describes triiodothyronine as a constituent of plasma, and suggests that on the basis of its high goiter-inhibiting property this may be the peripheral form of thyroid hormone. An orientation of this and other iodinated compounds recently described in blood and tissues by radiopaper chromatography to the events described in this paper would be of some interest.

SUMMARY

Immature male rats were injected with radioactive l-thyroxine in physiologic quantities. There was an immediate distribution of the injected material in the blood (38 per cent of the dose), liver (30 per cent), and the remaining tissues of the body (32 per cent). After this initial distribution, rapid diffusion occurred into the gastrointestinal tract, chiefly by way of bile but probably also by direct secretion. At equilibrium the gastrointestinal tract, consisting of stomach and small and large bowel, contained at any time about one half of the circulating radiothyroxine or intermediates thereof. A massive recirculation of radioactivity occurred from the bowel, presumably via the portal and lymphatic drainage. The rapidity of the recirculation phase is emphasized by the disparity between the rate at which radioactivity was secreted into the bowel, more than 100 per cent per hour, and the rate at which it left the bowel in the feces, about 3 per cent per hour. Two thirds of injected radioactivity was ultimately excreted in feces and one third in urine. Thyroxine, or some derivative thereof, was slowly removed from blood by fixation in tissues, particularly the liver. Such fixation also occurred in kidneys and in the tissues at large at a rate of about 1 per cent per hour. More than one half of the residual radioactivity was in the liver 16 days following injection.

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THE CAPACITY OF THE ADRENALECTOMIZED RAT TO SECRETE HYDROGEN AND AMMONIUM IONS[1]

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IT IS generally recognized that reductions in sodium and chloride concentration and increase in potassium concentration constitute major abnormalities in plasma composition in adrenal insufficiency (Loeb 1932; Harrop *et al.*, 1933, 1936). Somewhat less generally appreciated but no less significant are reductions in plasma pH and bicarbonate concentration, indicative of a metabolic type of acidosis (Swingle and Wenner, 1927; Hastings and Compere, 1931). These findings imply that deficiencies exist in renal mechanisms for regulation of acid base balance, as well as in those concerned with regulation of total electrolyte concentration (Jimenez-Diaz, 1936). It seemed profitable therefore to investigate the capacity of the adrenalectomized animal to excrete titratable acid and ammonia under normal conditions and under conditions of acid stress. It has been observed that these renal responses are deficient in either circumstance, facts which may account for chronic acidosis and poor tolerance to acid stress in the adrenalectomized animal. They likewise provide a rational explanation for the observation that adrenalectomized animals are maintained in a better state of health when they are provided with available base in the form of sodium lactate or bicarbonate than when they are maintained on sodium chloride alone (Allers, 1935).

METHODS

Experiments were performed on female Sprague-Dawley rats weighing from 200 to 250 grams. Animals were bilaterally adrenalectomized in one stage and were allowed a recovery period of 7 to 10 days prior to use. They were maintained on a high salt diet without further supportive therapy. During the course of each experiment, all animals were kept in wire mesh metabolism cages so constructed as to prevent contamination of the urine with food or feces. Each rat was weighed daily and the daily food and fluid intake and urine output recorded. Acid loading was accomplished by administration of ammonium chloride by stomach tube as a 5% solution. In those experiments in which the animals were maintained on a sodium free diet, food of the following composition was

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fed ad lib: casein, 18%; sucrose, 71%; Wesson oil, 5%; cod liver oil 2%; salts 4%; plus 12 cc. of vitamin solution in alcohol sprinkled over each 2000 grams of ration. The salt mixture was composed of $K_2HPO_4 \cdot 3H_2O$ 46%; $CaHPO_4$ 8%; $MgSO_4$ 11%; $CaCO_3$ 32%; and iron citrate 3%. The fact that the adrenalectomized animals succumbed when placed on a sodium free diet is indicative of the completeness of the operative procedure for all of the normal animals survived this test procedure without loss of weight.

Urinary pH was determined with a Cambridge glass electrode, titratable acid by electrometric titration of the urine to a pH of 7.40. urinary ammonia by Conway's micro diffusion method, chloride by Sendroy's modification of the method of Van Slyke and Heller, and sodium and potassium by an internal standard flame photometer.

RESULTS

In order to determine whether the renal mechanisms for the excretion of ammonia and titratable acid are adversely affected by adrenalectomy, the responses of a series of 12 normal and 11 adrenalectomized rats were compared under conditions of acid loading. Following a three day control period, either 50 or 100 mg. of ammonium chloride were administered on each of five successive days. A two day recovery period completed the

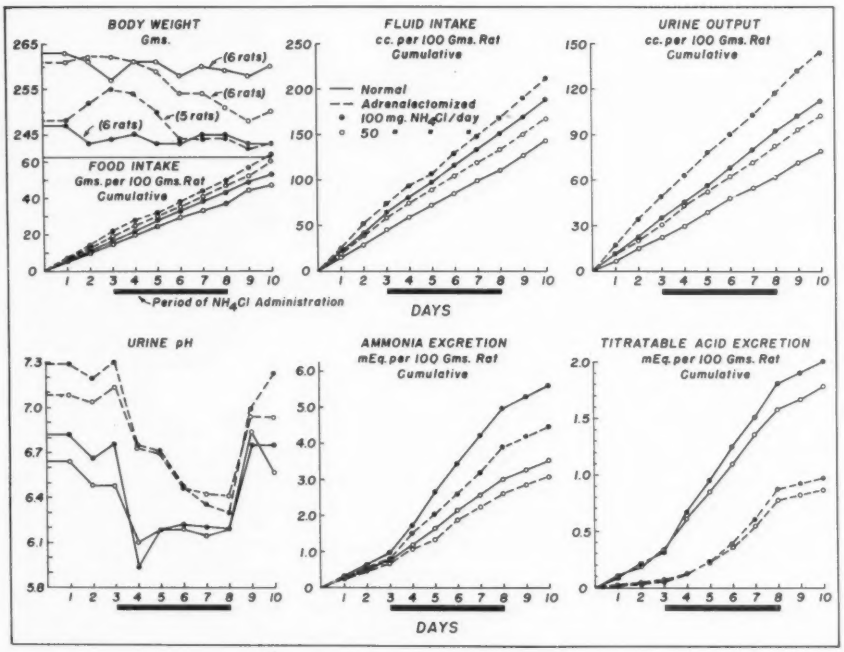

FIG. 1. Effects on ammonia and titratable acid output of the oral administration of ammonium chloride in dosage of 50 and 100 mg. per day to normal and adrenalectomized rats.

experiment. During the course of these experiments, the animals were allowed free access to food and to 0.9% NaCl solution and tap water.

All animals, both normal and adrenalectomized, survived acid loading. As is evident in Figure 1, the normal animals maintained essentially constant body weight whereas the adrenalectomized animals lost approximately 10 grams during the period of acid loading in spite of greater food

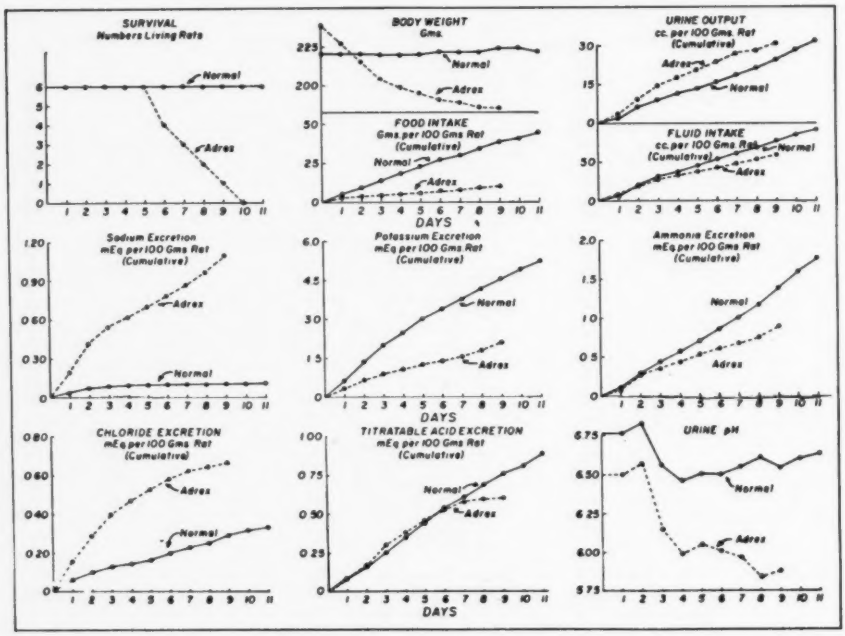

FIG. 2. Effects of sodium-free diet on the excretion of several electrolytes by normal and adrenalectomized rats.

and fluid intake. The characteristic differences in renal response to acid loading in normal and adrenalectomized animals are apparent in the lower half of Figure 1. Prior to acid loading, urine pH is significantly higher in the adrenalectomized animals than in the normals. With the imposition of an acid load, urine pH decreases to nearly the same level in both groups but much more promptly in the normals. This fact is evident not only with the low but also with the high dose levels of ammonium chloride. With recovery, there is a return of urine pH to control levels.

These differences in urine pH between operated and control animals are reflected in differences in rates of titratable acid excretion. Thus, during the initial control period the rate of excretion of titratable acid by

the normal animals is roughly 5 times that of the adrenalectomized. Although, the rate on the eighth day of acid loading is almost as great in the adrenalectomized as in the control animals, the approach to this maximum is much slower so that a marked deficit is apparent.

In contrast, although differences in rates of excretion of ammonia are

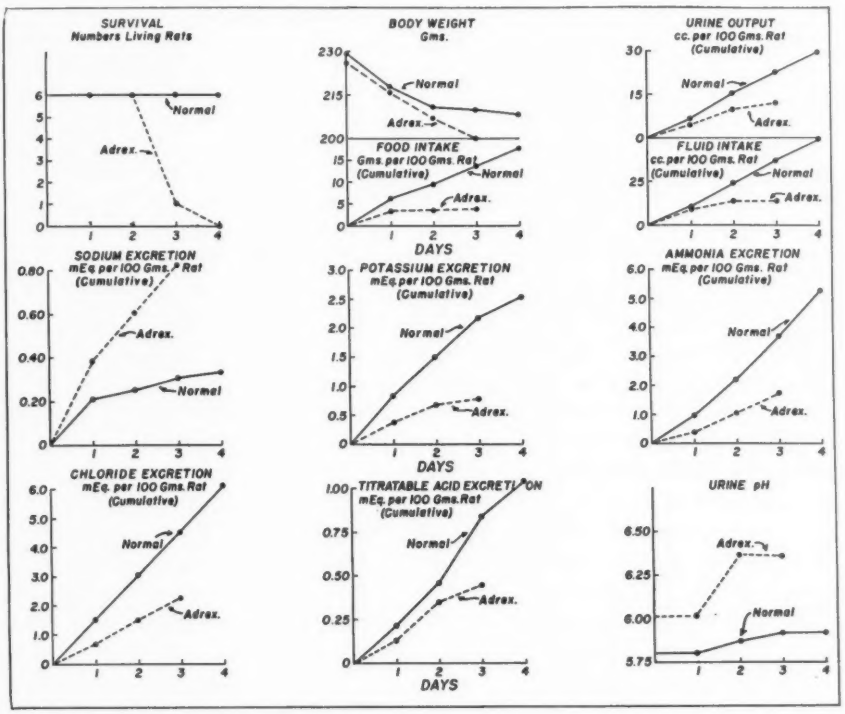

FIG. 3. Effects of sodium-free diet and the oral administration of 100 mg. ammonium chloride on the excretion of several electrolytes by normal and adrenalectomized rats.

less evident during the three control periods, the adrenalectomized animals at no time during the five day period of acid loading attain a rate of ammonia excretion equivalent to that of the normals. Thus, the deficit in ammonia output by the adrenalectomized animal averages 64 microequivalents per day per 100 grams of rat at the lower acid load and 177 microequivalents at the higher acid load. Such a constant deficit must lead to a progressively increasing depletion of base reserves.

It is apparent that the adrenalectomized animal is capable under acid stress of decreasing urine pH and increasing rates of excretion of titratable

acid and ammonia. However, the magnitudes of these renal responses are less than in the normal animal.

Since the adrenalectomized animals in the experiments just described were able to handle an acid load of as much as 2 milliequivalents per day for 5 days when supplied with adequate quantities of sodium chloride in their diet, it was necessary to limit salt intake in order to demonstrate the relationship of the renal defect in ammonia and titratable acid excretion to survival. Two series of experiments summarized in Figures 2 and 3 were performed on animals maintained on a sodium-free diet. In the experiments summarized in Figure 2 only sodium restriction was imposed. In those summarized in Figure 3, both sodium restriction and an acid load of 100 mg. of ammonium chloride per day were imposed.

As is apparent in Figure 2, all normal animals survived 11 days on the regimen of salt restriction without change in body weight. When in addition an acid load was administered (cf. Figure 3) weight loss occurred most strikingly during the first 2 days. On the other hand the adrenalectomized rats on salt restriction alone (cf. Figure 2) survived 7 days on an average, losing 38 grams or 16% of body weight during this interval. When in addition an acid load was imposed (cf. Figure 3) survival was reduced to an average of $2\frac{1}{2}$ days and weight loss amounted to 12%.

In both series of experiments food intake of the adrenalectomized animals was far less than that of the normals. Fluid intake likewise was less. Unfortunately no compensation for these differences was made by pair feeding and pair fluid administration. However, food and fluid intake in the adrenalectomized rats subjected to salt deprivation was essentially the same as in those subjected to salt deprivation and acid loading. Hence, food and fluid intake were not the significant factors determining the difference in times of survival of these two groups.

By far the most striking abnormality of the adrenalectomized animal subjected to sodium deprivation is the maintained high rate of urinary sodium and chloride loss (cf. Figure 2). The normal animal quickly reduced sodium loss to about 25 microequivalents per day. The deficit in potassium excretion in the adrenalectomized animal is probably of little significance in view of the limited intake of food. Although ammonia output is significantly less in the adrenalectomized than in the normal animal the output of titratable acid is nearly the same and the urine pH distinctly lower. It is probable that these adrenalectomized animals, in consequence of their reduced food intake, were in ketosis and that the comparison in Figure 2 is one between normal animals with normal acid base balance and operated animals in at least moderate acidosis.

When in addition to this endogenous acid load a load of 2 milliequivalents of ammonium chloride (100 mg.) per day was added (cf. Figure 3)

the rate of sodium loss was accelerated in comparison with that exhibited by animals subjected to salt deprivation alone (cf. Figure 2). Furthermore, the imposition of a high exogenous acid load makes the normal and adrenalectomized animals more nearly comparable with respect to acid base relationships. Hence, the expected deficiencies in ammonia and titratable acid output and incapacity to lower urine pH described above for the adrenalectomized animals are apparent in the experiments summarized in Figure 3.

DISCUSSION

It is probable that a number of distinct tubular mechanisms are concerned with the reabsorption of sodium from the glomerular filtrate. These include at least two in the proximal segment concerned with the reabsorption of sodium in association with chloride and with bicarbonate. Evidence of Walker *et al.*, (1937) and Wesson *et al.*, (1948) suggest that these two mechanisms are independent. A third mechanism, concerned with the absorption of sodium and chloride and capable of transporting these ions against a concentration gradient, is localized in the distal segment (Walker *et al.*, 1941). No less than three exchange processes, presumably carried on by the distal tubule, are also concerned with sodium absorption; namely, the processes of potassium-sodium (Berliner and Kennedy, 1948), hydrogen-sodium, and ammonium-sodium exchange (Pitts and Alexander, 1945). To what extent these processes represent activities of separate mechanisms or separate activities of a single mechanism is presently uncertain. Data presented above indicate that the latter two processes are performed less effectively in the adrenalectomized than in the normal animal. Failure to salvage adequately fixed base from the tubular urine in exchange for hydrogen ions or ammonium ions, results not only in increased loss of base but in a disturbance of body acid base balance. Thus, base which normally would be returned to the body as bicarbonate is lost in the urine.

It should be emphasized that the deficiencies in the ammonia and acid exchange processes are relative rather than absolute, for the kidney of the adrenalectomized animal responds to acid stress, though less effectively than does that of the normal animal, with an increase in acid and ammonia output. The magnitude of the disturbance in acid base balance will depend on the acid load. When maintained on an alkaline diet, little disturbance in acid base balance would be expected. Furthermore, deficiencies in these exchange processes probably account for only a fraction of the total loss of base in adrenal insufficiency.

Further work directed toward an elucidation of the nature of the disturbances in the acid base regulatory mechanisms is in progress.

SUMMARY

The capacity of the kidney of the adrenalectomized rat to excrete ammonia and titratable acid is diminished under conditions of moderate or severe acid stress. This deficiency no doubt underlies the lowering of bicarbonate reserves commonly observed in adrenal insufficiency.

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THE ROLE OF THE PITUITARY-ADRENAL SYSTEM IN CYSTINE-S^{35} INCORPORATION INTO PROTEIN[1]

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STUDY of the role of the pituitary-adrenal axis in protein metabolism is desirable in view of the conflicting reports of Novack (1952) and of White *et al.* (1948), Friedberg (1950) and Marshall and Friedberg (1951). Novack showed the administration of adrenocorticotrophin (ACTH) to increase C^{14}-histidine incorporation into the visceral and muscle protein of the intact mouse. The other authors, in studies employing C^{14}-glycine or S^{35}-methionine, showed adrenalectomy to increase incorporation into the tissues of the fasted mouse and the administration of dehydrocorticosterone to the adrenalectomized rats or ACTH to the hypophysectomized rat to suppress amino acid incorporation.

In an attempt to resolve this conflict we have studied the role of the pituitary-adrenal axis in the incorporation of S^{35}-cystine into tissue proteins of the albino rat.

METHODS

Equal numbers of male and female Sprague-Dawley rats were used in all experiments and were kept on an *ad libitum* regime of Purina diet at all times. The hypophysectomized rats were obtained from the Hormone Assay Laboratories, Chicago, Illinois and were kept on the stock diet for one week prior to use.

Table 1 presents information concerning the endocrine treatment, average weight, and size of the various groups of animals. The ACTH was calculated in terms equivalent to the Armour LA-1-A standard. Purified growth hormone was generously supplied by Dr. C. H. Li of the University of California. The adrenocortical extract (ACE) was the aqueous Upjohn preparation, cortisone acetate (CA) was a gift of Merck & Co. and the desoxycorticosterone acetate (DCA) was obtained commercially.

On the last day of the experimental period a standard dose of S^{35}-labeled DL-cystine[2] was administered intraperitoneally and the animals sacrificed 8 hours later. Heart, liver, kidneys, spleen, intestinal mucosa and plasma were rapidly removed and stored at $-17°$ C. The analytical procedure was as follows: proteins were precipitated with trichloracetic acid, washed, reduced with β-mercaptoethanol and the sulfur converted to

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[2] The S^{35}-labeled DL-cystine was synthesized by Dr. H. Tarver, University of California, Berkeley.

sulfate by wet oxidation. The sulfate was precipitated as the benzidine derivative; the S^{35} of this preparation was assayed with an end-window Geiger-Mueller tube and the total sulfate determined by titration. The results are expressed as specific activity (counts/minute/μmole of sulfur), corrected for body weight and radioactive decay. A detailed discussion of analytical methods as well as the expression of results has been published (Lee, Anderson, Miller and Williams, 1951).

TABLE 1. DESCRIPTION OF TREATMENT OF EXPERIMENTAL GROUPS

Group	Treatment				Average weight, grams	Number in group
	Hormone	Dose	Days	Route of Administration		
Normal	None	—	—	—	245	4–12
Normal	ACTH	1 mg./day	3	Intraperitoneal	180	5
Normal	Growth	1 mg./day	7	Intraperitoneal	160	6
Normal	Insulin	4 units/day	3	Subcutaneous	220	5
Orchidectomy	None	—	—	—	220	11
Hypophysectomy	None	—	—	—	170	9
Hypophysectomy	ACTH	1 mg./day	7	Intraperitoneal	170	6
Hypophysectomy	Growth	1 mg./day	7	Intraperitoneal	180	5
Adrenalectomy	None	—	—	—	190	6
Adrenalectomy	ACE	0.3 ml./day	3	Subcutaneous	205	10
Adrenalectomy	CA	0.5 mg./day	3	Subcutaneous	225	10
Adrenalectomy	DCA	0.5 mg./day	3	Subcutaneous	210	7

RESULTS

I. *The Intact Rat*

Administration of ACTH to the normal rat resulted in highly significant increases in cystine-S^{35} incorporation into heart, liver, spleen and plasma protein ($P < 0.01$ or 0.001, Table 2). The increase with respect to kidney had no statistical significance ($P > 0.05$) and with respect to intestinal mucosa only doubtful significance ($P < 0.02$). Consequently, these results are in accord with Novack's studies using intact mice (1952).

TABLE 2. THE EFFECT OF EXOGENOUS ACTH, GROWTH HORMONE AND INSULIN, AND OF ORCHIDECTOMY ON THE INCORPORATION OF CYSTINE-S^{35} INTO THE TISSUE PROTEINS

	Specific activity of tissue protein				
	Control	ACTH	GH	Insulin	Orchex.
Heart	0.69 ± 0.03	1.10 ± 0.07	0.69 ± 0.03	0.87 ± 0.02	0.63 ± 0.02
Liver	1.18 ± 0.04	1.83 ± 0.13	1.08 ± 0.02	1.44 ± 0.08	0.79 ± 0.04
Kidney	3.28 ± 0.27	4.30 ± 0.35	2.47 ± 0.13	3.75 ± 0.28	2.39 ± 0.11
Spleen	2.02 ± 0.10	3.10 ± 0.19	2.52 ± 0.16	2.48 ± 0.09	1.93 ± 0.07
Intestinal Mucosa	4.31 ± 0.27	5.71 ± 0.29	4.72 ± 0.40	5.07 ± 0.26	5.31 ± 0.46
Plasma	2.58 ± 0.34	5.41 ± 0.31	3.51 ± 0.22	3.93 ± 0.79	2.58 ± 0.11

Since an increase in labeling, under these or similar conditions, has been interpreted to mean an increase in the rate of protein synthesis (White *et al.*, 1948; Friedberg and Greenberg, 1948), this result is inconsistent with

the view that adrenocortical stimulation is associated with protein breakdown. However, if this interpretation of isotopic data were correct, then increases should be found after growth hormone or insulin treatment and decreases after orchidectomy. The results of such studies are presented in Table 2. The administration of growth hormone resulted in a typical picture of growth (Li and Evans, 1948), the somatic increment (17.7%) being accompanied by a disproportional liver increment (21.3% greater than normal). However, with respect to cystine-S^{35}, changes of little or no statistical significance were noted; incorporation was greater than control for spleen and less for kidney ($P < 0.02$). After insulin injection incorporation was increased only with respect to liver and spleen ($P < 0.01$); after orchidectomy incorporation was decreased only with respect to kidney and liver ($P < 0.01$–0.001). Consequently, the interpretation that an increase in labeling reflects an increase in the rate of protein anabolism is an oversimplification of the significance of this type of data.

II. *The Hypophysectomized Rat*

In the hypophysectomized rat incorporation of cystine-S^{35} was significantly reduced with respect to all tissues studied ($P < 0.01$ or < 0.001) with the exception of intestinal mucosa ($P < 0.20$) (Table 3). ACTH

TABLE 3. THE EFFECT OF ADMINISTRATION OF ACTH AND OF GROWTH HORMONE ON THE INCORPORATION OF CYSTINE-S^{35} INTO THE TISSUE PROTEIN OF THE HYPOPHYSECTOMIZED RAT

	Specific activity of tissue protein			
	Control	Hypex.	Hypex. & ACTH	Hypex. & GH
Heart	0.69 ± 0.03	0.41 ± 0.04	0.92 ± 0.04	0.88 ± 0.07
Liver	1.18 ± 0.04	0.50 ± 0.03	1.37 ± 0.26	1.19 ± 0.11
Kidney	3.28 ± 0.27	1.56 ± 0.10	2.87 ± 0.15	2.54 ± 0.21
Spleen	2.02 ± 0.10	1.28 ± 0.09	2.70 ± 0.31	2.64 ± 0.20
Intestinal Mucosa	4.31 ± 0.27	3.81 ± 0.38	4.89 ± 0.41	6.11 ± 1.48
Plasma	2.58 ± 0.34	1.06 ± 0.16	3.66 ± 0.19	3.13 ± 0.20

administration resulted in analytical values returning toward normal, actually exceeding control values in all cases, with the exception of intestinal mucosa. Growth hormone also produced similar effects.

In several of the studies the protein specific activity of the pooled adrenals was determined (Table 4). In the normal animal ACTH administration resulted in lower adrenal values than found for the control series. The significance of the decrease cannot be assessed because of the unavailability of replicate determinations. In the hypophysectomized animal the adrenals were atrophic and absolutely no detectable cystine-S^{35} had been incorporated into gland protein whereas in the case of the hypophysectomized ACTH-treated animal the glands were larger, normal-appearing

TABLE 4. THE EFFECT OF ACTH AND OF GROWTH HORMONE ON THE INCORPORATION OF CYSTINE-S^{35} INTO THE ADRENAL PROTEIN OF THE INTACT AND HYPOPHYSECTOMIZED RAT

	Specific activity of adrenal protein		
	Treatment		
	Control	ACTH	G.H.
Intact	1.76	0.60	1.21
Hypophysectomy	0.00	1.83	1.35

and significant quantities of cystine-S^{35} had been incorporated into gland protein. A surprising finding was the adrenal stimulus provided by growth hormone. These glands very much resembled those in the previously mentioned series and the processes for the incorporation of cystine-S^{35} into adrenal protein had definitely been restored. It is worthy of mention that Selye (1951) has adduced evidence showing that growth hormone appears to exert a direct effect on "mineralo-corticoid" production.

III. *The Adrenalectomized Rat*

Adrenalectomy resulted in diminished cystine-S^{35} incorporation into the protein of heart, liver, kidney and spleen ($P < 0.02$–0.001) (Table 5). Maintenance of the adrenalectomized animal on aqueous adrenocortical extract, cortisone acetate, or desoxycorticosterone acetate, resulted in values equal to, or greater than, control. It is of interest to note that intestinal mucosa showed a significant response neither to adrenalectomy nor to steroid replacement therapy. Furthermore, although adrenalectomy resulted in no significant changes with respect to plasma protein, steroid replacement in all cases produced highly significant increases in cystine-S^{35} incorporation into plasma protein ($P < 0.01$–0.001).

TABLE 5. THE EFFECT OF ADRENOCORTICAL EXTRACT, CORTISONE ACETATE AND DESOXYCORTICOSTERONE ACETATE ON THE INCOPORATION OF CYSTINE-S^{35} INTO THE TISSUE PROTEIN OF THE ADRENALECTOMIZED RAT

	Specific activity of tissue protein				
	Control	Adrex.	Adrex. & ACE	Adrex. & CA	Adrex. & DCA
Heart	0.69 ± 0.03	0.43 ± 0.04	0.82 ± 0.03	0.85 ± 0.02	0.95 ± 0.05
Liver	1.18 ± 0.04	0.81 ± 0.19	1.35 ± 0.13	1.29 ± 0.04	2.19 ± 0.41
Kidney	3.28 ± 0.27	2.00 ± 0.24	3.81 ± 0.39	4.08 ± 0.26	4.17 ± 0.38
Spleen	2.02 ± 0.10	1.49 ± 0.10	3.14 ± 0.28	2.96 ± 0.17	3.19 ± 0.34
Intestinal Mucosa	4.31 ± 0.27	6.26 ± 0.94	5.11 ± 0.48	5.97 ± 0.26	4.95 ± 0.70
Plasma	2.58 ± 0.34	2.09 ± 0.24	3.80 ± 0.23	4.17 ± 0.34	3.50 ± 0.32

DISCUSSION

The experiments reported here provide five observations concerning the role of the pituitary-adrenal axis in the incorporation of cystine-S^{35} into

the tissue protein of the fed albino rat. (1) The administration of ACTH to the intact rat increased cystine-S^{35} incorporation. (2) Hypophysectomy reduced cystine-S^{35} incorporation. (3) Adrenalectomy reduced cystine-S^{35} incorporation. (4) Administration of ACTH to the hypophysectomized rat restored cystine-S^{35} incorporation to control values. (5) Maintenance of the adrenalectomized rat with various adrenal steroid preparations restored cystine-^{35} incorporation to control values. Consequently it can be concluded that the pituitary-adrenal system plays a positive role in the processes responsible for the incorporation of radiocystine into protein.

Interpretation of these findings in terms of *net* protein synthesis or *net* protein breakdown is, however, not warranted. Suggestive evidence has been presented that the mere increase in radioamino acid incorporation at a given time after administration cannot be interpreted *a priori* as an increase in protein anabolism. It is probable that the converse also follows, that diminished incorporation is not consonant with an anti-anabolic, or catabolic, effect. An alternative hypothesis exists, namely that increases or decreases in incorporation reflect similar changes in protein turnover rates. Support of this hypothesis will await studies similar to those presented here, but executed in such a manner as to obtain detailed kinetic data.

A discrepancy exists between the work reported by Novack (1952) and in this paper, on the one hand, and that reported by White *et al.* (1948), Friedberg (1950) and Marshall and Friedberg (1951) on the other hand. These latter workers have brought forth evidence supporting the conclusion that the pituitary-adrenal system plays a negative role in the incorporation of radio-amino acids into protein. It should be noted, however, that in the report of White *et al.* studies employing fed adrenalectomized controls were not presented. This is of importance in view of the reports of Engel and coworkers (1949 a and b) showing that dietary factors block amino acid and protein catabolism in the rat. Furthermore, in the work of Friedberg (1950) and Marshall and Friedberg (1951) large doses of hormones were used. Ingle and Prestrud (1949) have shown that the administration of small doses of adrenocortical extract to adrenalectomized rats results in greater nitrogen retention and somatic growth than those receiving saline only whereas larger doses resulted in greater nitrogen loss and suppression of somatic growth.

One further point needs mention. The data presented in Table V show, contrary to expectation, that DCA exhibits a distinct positive effect on radio-cystine incorporation into protein. Furthermore, with respect to this parameter of cortical function, DCA appears to be equally effective as CA. This observation is not in agreement with the concept of the dis-

creteness of action of the "glucocorticoids" and the "mineralocorticoids." Recently, several reports have appeared showing the rigid classification of adrenal steroids into those effecting salt-water metabolism (mineralocorticoids) and those effecting carbohydrate-protein metabolism (glucocorticoids) to be untenable. Overman *et al.* (1951) have shown CA to antagonize the action of DCA on salt-water metabolism in the dog. Luft and Sjögren (1951) have shown, in the human, that CA promotes Na retention and K loss as well as DCA. Whether the action of DCA on cystine-S^{35} incorporation is direct or the result of changes in salt-water metabolism cannot be said as yet. However, it should be pointed out that Hayano and Dorfman (1951) have shown desoxycorticosterone to affect the activities of various enzymes concerned with amino acid and protein metabolism.

SUMMARY

The role of the pituitary-adrenal system in the incorporation of cystine-S^{35} into protein was studied in the fed albino rat. Incorporation was reduced following hypophysectomy or adrenalectomy and was restored by administration of adrenocorticotrophin in the former case and various adrenal steroid preparations in the latter case. With respect to the adrenalectomized rat, aqueous adrenocortical extract, cortisone acetate and desoxycorticosterone acetate were all capable of restoring incorporation to control levels. Administration of adrenocorticotrophin to the intact rat resulted in increased cystine-S^{35} incorporation into protein.

Data are presented concerning the effects of growth hormone, insulin and orchidectomy on the incorporation of cystine-S^{35} into protein.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be limited to ten minutes, should send FOUR COPIES of the title and abstract to the Vice-President, Dr. Lawson Wilkins, Johns Hopkins Hospital, Baltimore 5, Maryland, not later than FEBRUARY 1, 1953. IT IS IMPERATIVE THAT THE ABSTRACTS BE INFORMATIVE AND COMPLETE WITH RESULTS AND CONCLUSIONS IN ORDER THAT THEY MAY BE OF REFERENCE VALUE AND SUITABLE FOR PRINTING IN THE PROGRAM AND JOURNALS OF THE SOCIETY. THE FOLLOWING REGULATIONS FOR THE PREPARATION OF ABSTRACTS AND TITLES MUST BE CAREFULLY FOLLOWED TO INSURE CONSIDERATION OF THE PAPER FOR THE PROGRAM:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-

authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was

unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

ANNOUNCEMENT

The University of Minnesota will present a continuation course in "Recent Advances in Diagnosis for Internists" at the Center for Continuation Study on February 16 to 18, 1953. Although intended primarily for specialists in internal medicine, the program will be of interest to many pediatricians and general physicians. This course will provide information concerning techniques which have been introduced or developed within the past five to ten years which have increased our knowledge of basic physiology and which are becoming or give promise of becoming standard diagnostic procedures. Recent developments in the fields of cardiology, respiratory disease, renal disease, hematology, gastro-enterology, and endocrinology will be emphasized.

Under the direction of Dr. C. J. Watson, Professor and Director of the Department of Medicine, an outstanding guest faculty has been assembled: Dr. Carl V. Moore, Professor, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri; Dr. Thomas E. Machella, Assistant Professor, Department of Medicine, and Associate in Physiology, University of Pennsylvania School of Medicine, and Chief of Gastro-Intestinal Clinic, Hospital of the University of Pennsylvania, Philadelphia; Dr. Robert P. Grant, U. S. Public Health Service Hospital, Baltimore; and Dr. William W. Engstrom, Associate Professor, Department of Medicine, Marquette University School of Medicine, and Director, Metabolism Section, Milwaukee County Hospital. The remainder of the faculty will include clinical and full-time members of the staff of the University of Minnesota Medical School and the Mayo Foundation.

Lodging and meal accommodations are available at the Center for Continuation Study.